

CHARACTERIZATION OF THE GnRH- INDUCED CORPUS LUTEUM IN THE CYCLING HEIFER

SHEILA MARGARET RUSBRIDGE
BVSc (Bristol)

Thesis submitted to the University of Edinburgh for the degree of
Doctor of Philosophy

The Roslin Institute,
Department of Reproduction & Development,
Roslin,
Midlothian,
EH25 9PS;

and

The University of Edinburgh,
Department of Obstetrics & Gynaecology,
Centre for Reproductive Biology,
37 Chalmers Street,
Edinburgh,
EH3 9EW;

and

The University of Edinburgh,
Department of Veterinary Clinical Studies,
Easter Bush,
Roslin,
Midlothian,
EH25 9RG.

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ABSTRACT

Although recent studies in other laboratories have greatly increased our understanding of luteal function in cattle, we are still unable to accurately synchronize luteolysis, preovulatory follicular growth and ovulation with commercially available pharmacological preparations. The aim of these studies was to investigate the factor(s) which controls the formation, function and regression of the GnRH-induced corpus luteum (CL).

Initially, we sought to develop an experimental model to examine the characteristics of the CL induced in intact heifers with normal oestrous cyclicity, by GnRH injection in the early luteal phase. Administration of GnRH on Day 6 after the synchronized oestrus resulted in ovulation and formation of an additional CL in >70% of animals. Following prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in the mid-luteal phase, the spontaneously-formed CL underwent luteolysis while the induced CL did not, leading to a delay in return to oestrus associated with a persistence of luteal function.

Having demonstrated the ovulatory competence of the dominant follicle of the first follicular wave, and the formation of a functional CL, it was of interest to examine the reason for the premature demise of the induced CL in the GnRH responders when compared to the spontaneously-formed CL. Administration of steroid-stripped bovine follicular fluid (bFF), which suppressed the growth of the dominant follicle, and reduced serum oestradiol concentrations, resulted in a further delay in the return to oestrus and a significant extension in the lifespan of the induced CL, when compared to the untreated responders, and provided circumstantial evidence that oestradiol was the endogenous agent responsible for regression of the induced CL.

Although luteal lifespan was extended by the use of bFF, the function of the induced CL was still limited compared to the spontaneously-formed CL. A comparison of the structure and composition of the induced CL with the spontaneously-formed CL of the same age, revealed no differences in concentrations of progesterone and oxytocin 7 days after formation, but a significant increase in LH receptor concentration in the induced CL. This suggests that the limitation in lifespan of the induced CL was not due to a lack of luteotrophic support. Furthermore, a subsequent study examining the interaction between the induced CL and the uterine endometrium, demonstrated that the GnRH responders with an induced CL were competent to respond to an oxytocin challenge with a 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ (prostaglandin F metabolite) response of similar magnitude to animals with a spontaneous CL. The similarity in response suggested that the premature demise of the induced CL was not the result of advanced release of $PGF_{2\alpha}$, or of oxytocin-induced reduction in luteotrophic support.

To investigate the possibility of an additional direct effect of bFF on the induced CL, the development of a simple procedure for the dispersion and culture of bovine luteal cells in our laboratory, enabled a study of the effect of bFF on luteal cell function *in vitro*. The study demonstrated that bFF contained a factor(s) that inhibited progesterone production by mixed populations of cultured luteal cells *in vitro*. The inhibitory effect was seen in both steroid-stripped, inhibin-enriched and inhibin-depleted fractions of bFF. Thus, bFF appears to inhibit luteal function *in vitro* by a direct effect on the CL, but extend luteal function *in vivo* indirectly by suppression of follicular growth and a reduction in peripheral oestradiol concentrations.

Finally, we compared the steroidogenic capacity *in vitro*, of dispersed luteal cells from induced and spontaneous CL 7 days after ovulation, in response to treatment with LH (the primary luteotrophin in the bovine), and dibutyryl cyclic adenosine 3',5'-monophosphate (dbcAMP), which is independent of receptor integrity. Both spontaneous and induced dispersed cells were non-responsive to LH, but spontaneous luteal cells dramatically increased progesterone production in the presence of dbcAMP, suggesting that dispersion had damaged the LH receptor. Progesterone production in response to dbcAMP by luteal tissue from induced CL was markedly reduced, suggesting a failure of the steroidogenic response at an as yet unidentified point in the second messenger pathway.

Collectively, we have demonstrated that administration of GnRH in the early luteal phase results in formation of an additional CL of limited functional capacity. The premature demise of the induced CL appears to be a consequence of an endogenous signal, which is probably oestradiol, from the dominant follicle. This functional association between luteal and follicular elements in the ovary is a target for future research attempting to improve oestrous synchronization techniques in cattle.

DECLARATION

I hereby declare that this thesis has been composed by myself, and has not been submitted for any other degree, in Edinburgh or elsewhere. The work presented herein is my own, and all work of other authors is duly acknowledged. I also acknowledge all assistance given to me during the planning, execution and presentation of this thesis.

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PUBLICATIONS ARISING FROM THE THESIS

a. ORAL COMMUNICATIONS

1. Rusbridge, S.M. & Webb, R. (1991) Control of the lifespan of corpora lutea induced during the early luteal phase of the oestrous cycle in heifers *J. Reprod. Fert. Abst. ser. No. 7*, abst. 54.
2. Rusbridge, S.M., Bramley, T.A. & Webb, R. (1991) Further studies on the control of the lifespan of corpora lutea (CL) induced during the early luteal phase of the oestrous cycle in heifers *J. Reprod. Fert. Abst. ser. No. 8*, abst. 31.
3. Rusbridge, S.M., Bramley, T.A. & Webb, R. (1992) A comparison of GnRH-induced corpora lutea (CL) and spontaneously formed CL in heifers *J. Reprod. Fert. Abst. ser. No. 9*, abst. 33.

b. REVIEW ARTICLES

1. Webb, R., Gong, J.G., Law, A.S. & Rusbridge, S.M. (1992) Control of ovarian function in cattle *J. Reprod. Fert. Suppl.* **45** 141-156.

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INTRODUCTION

The failure to detect oestrus accurately in lactating dairy cows, and the consequent increase in the calving-to-conception interval, is a major cause of economic loss worldwide. The study of the reproductive physiology and endocrinology of cattle has led to the development of commercially viable pharmacological preparations for the control of the bovine oestrous cycle. However, the use of these products has not been adopted as standard practice, due to the variability in success rates. The opportunity to control luteal function and synchronize follicular growth would enable more precise oestrous synchronization regimes, leading to improved conception rates at artificial insemination. This series of studies was therefore carried out to provide a better understanding of luteal function and the factor(s) which can influence the lifespan of the corpus luteum.

CHAPTER 1

LITERATURE REVIEW: THE BOVINE CORPUS LUTEUM: FORMATION, FUNCTION AND REGRESSION

1.1. Historical

The first detailed description of the corpus luteum (CL) was published in 1672 by de Graaf (Short, 1977) who referred to "globular bodies" that formed in the place of "ova". He provided accurate drawings of the sheep CL, and noted that in the rabbit these "globules" are present only after ovulation. The term "corpus luteum" was introduced by Malpighi (Short, 1977), who took note that the cow ovary has a "yellow body" that is probably glandular in nature. The mysteries of the CL remained undisclosed for over two centuries until Prenant, in 1898, proposed that it was a gland of internal secretion. Frankel (1903) and Magnus (1901) separately published the first scientific evidence for the physiological role of the CL; cauterization of individual CL in rabbits resulted in abortion or resorption of the embryos, whereas in sham-operated control animals, pregnancies survived. Years later the active principle, progesterone, was identified as the primary endocrine secretory product of the CL (Corner & Allen, 1929) and purified and crystallized in the laboratory of Allen & Wintersteiner (1934).

1.2. General reproductive physiology of the cow

The reproductive system of the cow is fully developed at birth, and large antral follicles may be found in the ovaries of many prepubertal animals (Hammond, 1927; Casida, Chapman & Rupel, 1935; Perry & Rowlands, 1962; Desjardins & Hafs, 1969) although the final stages of follicular development, ovulation and the formation of luteal tissue only occur following the attainment of puberty which is usually reached at 9-15 months of age (Hammond, 1927; Moran, Quirke & Roche, 1989). The cow is a polyoestrous species; with the exception of pregnancy and the immediate post-partum period regular cyclic ovarian activity continues from sexual maturity throughout life. Unlike the sheep (Marshall, 1937) the cow is an aseasonal breeder.

1.3. The oestrous cycle

1.3.1. Temporal characteristics

Each oestrous cycle is characterized by a short period of intense sexual activity or oestrus followed by a much longer period of behavioural quiescence. The length of the oestrous cycle is approximately 20 days for heifers and 21 days for cows with possible variation between and within individual animals ranging from 17 to 24 days (Hafez & Sugie, 1963; Robinson, 1977; Robinson & Shelton, 1991). The oestrous cycle may be divided into two stages; the luteal phase of approximately 17 days duration and the shorter follicular phase of 3-4 days duration, with the day of oestrus usually designated as Day 0 of the oestrous cycle (Hunter, 1980). The mean length of oestrus is 15-18 hours with a range of 6-30 hours (Hammond, 1927; Gomes, 1978). Factors influencing the duration of oestrus include parity, season of

the year, stage of lactation, breed, nutrition and number of cows that are in oestrus at the same time (Trimberger, 1948; Wishart, 1972; Hurnik, King & Robertson, 1975; Gomes, 1978). There is an increased incidence of mounting activity during the night (Esslemont & Bryant, 1976). There is a great variation between individuals in the intensity of heat signs. As the cow approaches oestrus she tends to search for other cows in oestrus, with licking and sniffing of the perineum, chin-resting and attempts to mount. It is generally agreed that the most reliable criterion that a cow or heifer is in oestrus is that she will stand to be mounted (Williamson, Morris, Blood, Cannon & Wright, 1972; Foote, 1975). The oestrous animal is restless and more active; there is a reduction in the time spent eating, resting and ruminating, and frequently a drop in milk yield. There is a genital discharge of transparent mucus, the vulva is swollen and congested and there is a slight elevation of body temperature. For a short time after service the cow stands with a raised tail and arched back. The vulval discharge subsequently turns yellowish-white and at about 48 hours after heat there is a sanguineous discharge, the blood coming mainly from the uterine caruncles (Arthur, Noakes & Pearson, 1982). Ovulation is spontaneous and normally occurs 24-48 hours after the onset of oestrus (Hansel & Trimberger, 1952; Quirk, Hickey & Fortune, 1986) or 10-12 hours after the end of oestrus (Henricks, Dickey & Niswender, 1970; Schams, Schallenberger, Hoffman & Karg, 1977). The luteal phase is characterized by the presence of a functional CL on one ovary. Following luteolysis and the regression of the CL there is rapid development of usually a single preovulatory follicle in the short follicular phase.

1.3.2. Endocrinology of the oestrous cycle

The gonadotrophins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are synthesized and secreted by the anterior pituitary gland under the control of the decapeptide hormone,

gonadotrophin-releasing hormone (GnRH), secreted in a pulsatile fashion from the hypothalamus (Pierce & Parsons, 1981; Ryan, Keutmann, Charlesworth, McCormick, Millius, Calvo & Vutyavanich, 1987; Everett, 1988). FSH and LH are glycoproteins (Reichert, 1962) composed of α and β subunits; the α subunit is common to both hormones while the β subunit confers hormonal specificity (Butt, 1975).

The ovarian sex steroids are derived from cholesterol and fall into three groups; the progestins, androgens and oestrogens. The major progestin, androgens and oestrogen produced by the bovine ovary are progesterone, androstenedione and testosterone, and oestradiol-17 β respectively (Short, 1962). During the mid-luteal phase mean LH concentrations are relatively low, averaging 0.5-2.0 ng/ml (Rahe, Owens, Fleeger, Newton & Harms, 1980; Walters, Schams & Schallenberger, 1984; Price, Morris & Webb, 1987). LH pulses occur every 3-4 hours with an amplitude of 0.7-7.0 ng/ml (Walters *et al.*, 1984). After luteal regression LH pulse frequency increases to 1 pulse every 40 to 60 minutes while pulse amplitude has been reported to either slightly decrease (Rahe *et al.*, 1980), increase (Schallenberger, Schondorfer & Walters, 1985) or remain unchanged (Peters, 1985a). Approximately 60 hours after the start of luteal regression at the onset of oestrus (Swanson & Hafs, 1971) there is a dramatic increase in LH concentrations to 50-60 ng/ml (Black & Hansel, 1972; Walters & Schallenberger, 1984). The preovulatory surge lasts approximately 6 hours (Snook, Saatman & Hansel, 1971; Walters & Schallenberger, 1984) and is characterized by an increase in pulse frequency to 2 pulses/hour and an increase in LH pulse amplitude. After the preovulatory surge LH pulses are abolished for about 12 hours before returning to the luteal phase pattern of secretion (Walters & Schallenberger, 1984; Schallenberger *et al.*, 1985).

Due to the lack of a sufficiently specific, sensitive and reliable radioimmunoassay (RIA) for bovine FSH, reports concerning FSH profiles are inconsistent. Mean FSH concentrations are relatively low during the luteal phase (approximately 50 ng/ml; Walters *et al.*, 1984). There is a surge of FSH coincident with the preovulatory LH surge (Walters & Schallenberger, 1984; Schallenberger *et al.*, 1985) and a secondary rise of FSH starting 4-12 hours after the end of the LH surge and reaching a peak of 80-90 ng/ml within 24 hours (Dobson, 1978; Walters & Schallenberger, 1984). Some workers report a pulsatile manner of FSH secretion with a frequency of 1 pulse every 2 hours and an amplitude of about 17 ng/ml (Walters *et al.*, 1984).

Peripheral progesterone concentrations are low (below 0.5 ng/ml) at oestrus (Glencross, Munro, Senior & Pope, 1973), but rise from around Day 3 to reach a peak on Days 10-14. Thereafter they remain elevated, averaging 4.0 ng/ml in jugular plasma, until luteolysis (Donaldson, Bassett & Thorburn, 1970; Henricks, Dickey & Hill, 1971; Smith, Fairclough, Payne & Peterson, 1975) when progesterone concentrations fall rapidly, reaching 1.0 ng/ml within 24-36 hours (Schallenberger, Schams, Bullerman & Walters, 1984). Pulses of progesterone secretion have been observed at a rate of approximately 1 pulse every 2 hours (Walters *et al.*, 1984) whilst others have failed to identify such a pattern (Peters, 1985a).

Circulating oestradiol concentrations are low, averaging 4 pg/ml during the luteal phase, rising at the onset of luteolysis to reach a peak at oestrus (Hansel & Echterkamp, 1972; Shemesh, Ayalon & Lindner, 1972; Dobson & Dean, 1974; Walters & Schallenberger, 1984) but fall rapidly following the preovulatory surge. Two small peaks have been detected around Days 4-6 and Days 12-15 of the cycle (Shemesh *et al.*, 1972; Dobson & Dean, 1974; Ireland & Roche, 1987). Pulses of oestradiol secretion of

approximately 6 pg/ml are detectable in vena cava blood, with a similar frequency to LH pulses (Walters *et al.*, 1984).

There is evidence of a close relationship between ovarian steroids and proteins, and gonadotrophins during the oestrous cycle. Administration of exogenous oestradiol induces a surge-like release of LH and FSH in cattle and sheep (Hobson & Hansel, 1972; Swanson & McCarthy, 1978; Short, Randel, Staigmiller & Bellows, 1979; Butler, Katz, Arriola, Milvae & Foote, 1983; Peters, 1984) mediated initially through an increase in pituitary sensitivity to GnRH and subsequently by an increase in GnRH secretion from the hypothalamus (Kesner, Convey & Anderson, 1981; Schoenemann, Humphrey, Crowder, Nett & Reeves, 1985). Oestradiol increases mean LH concentration (Critser, Miller, Gunsett & Ginther, 1983) and LH pulse amplitude (Kinder, Garcia-Winder, Imakawa, Day, Zalesky, D'Occhio, Kittok & Schanbacher, 1983; Stumpf, Day, Wolfe, Wolfe, Clutter, Kittok & Kinder, 1988a) with a decrease in pulse frequency (Kinder *et al.*, 1983). In contrast, other studies report an increase in LH pulse frequency (sheep: Karsch, Foster, Bittman & Goodman, 1983), a decrease in LH pulse amplitude (sheep: Goodman & Karsch, 1980; cattle: Price & Webb, 1988) or a reduction in mean FSH and LH concentrations (sheep: Clarke, Funder & Findlay, 1982; Rawlings, Jeffcoate & Rieger, 1984; cattle: Butler *et al.*, 1983; Price & Webb, 1988). Active and passive immunization against oestradiol-17 β results in an elevation in plasma LH concentrations (Pant & Rawlings, 1973; Rawlings, Kennedy & Henricks, 1978; Webb, Land, Pathiraja & Morris, 1984).

Exogenous progesterone blocked the oestradiol-induced gonadotrophin surge in cattle (Hobson & Hansel, 1972; Roche & Ireland, 1981a; Schoenemann *et al.*, 1985). Progesterone has been reported to reduce tonic LH secretion (Price & Webb, 1988), LH pulse frequency (Ireland & Roche, 1982; Price & Webb, 1988) and mean FSH concentrations (Price &

Webb, 1988) or have no effect (Barnes, Kazmer, Bierley, Richardson & Dickey, 1980; Schoenemann *et al.*, 1985) in ovariectomized cattle. Removal of progesterone from the peripheral circulation by induction of luteolysis or withdrawal of a progesterone-releasing intravaginal device (PRID) increased LH pulse frequency and both LH and FSH pulse amplitude (Ireland & Roche, 1982). Immunization of ewes against progesterone resulted in an increase in LH pulse frequency (Thomas, Oldham, Hoskinson & Scaramuzzi, 1984).

Most reports conclude that androgens have little effect on the feedback control of gonadotrophin secretion (McCarthy & Swanson, 1976; D'Occhio, Kinder & Schanbacher, 1982; Butler *et al.*, 1983) although immunization of ewes against androstenedione increased LH pulse frequency (Martensz, Scaramuzzi & Van Look, 1979) and immunization of heifers against testosterone increased gonadotrophin concentrations (Price *et al.*, 1987).

The effects of progesterone and oestradiol are synergistic; administration of these steroids in physiological concentrations maintained normal concentrations of LH in ovariectomized sheep (Karsch, Legan, Ryan & Foster, 1980). Similar reports have been obtained for cattle (Price & Webb, 1988). Physiological concentrations of oestradiol and progesterone were capable of maintaining normal concentrations of FSH in short-term ovariectomized heifers (Price & Webb, 1988), although other studies reported a failure of steroid treatment to suppress FSH concentrations to levels in intact controls (sheep: Goodman, Pickover & Karsch, 1981; cattle: Roche & Ireland, 1981b) suggesting a role for other ovarian substances such as inhibin and related proteins, in the feedback control of gonadotrophin secretion. Inhibin is present in follicular fluid (de Jong, 1988). Replacement of ovarian steroids failed to suppress FSH concentrations in ovariectomized

ewes to within normal range (Goodman *et al.*, 1981) but a combination of steroids and follicular fluid was capable of reducing FSH concentrations to physiological levels (Martin, Price, Thiery & Webb, 1988). However, although follicular fluid treatment prevented the transient rise in FSH concentrations following unilateral ovariectomy in heifers (Johnson, Smith & Elmore, 1985) and suppressed peripheral FSH concentrations in intact cattle (Quirk & Fortune, 1986), other studies have reported no effect of follicular fluid on FSH concentrations (Johnson & Smith, 1985). Moreover, effects of steroid-free follicular fluid could still be demonstrated with inhibin-stripped follicular fluid (Law, Baxter, Logue, O'Shea & Webb, 1992). In ovariectomized ewes, bovine follicular fluid (bFF) has been reported to reduce peripheral LH concentrations (Findlay, Gill & Doughton, 1985; Clarke, Findlay, Cummins & Ewens, 1986).

1.4. Ovarian follicular and luteal dynamics

Folliculogenesis is the process whereby primordial follicles progress through various stages of development, culminating in either atresia or ovulation. The cow is born with approximately 150,000 primordial follicles, but this figure is reduced by natural wastage to approximately 3,000 by 15-20 years of age (Erickson, 1966). Following the initiation of ovarian function during foetal life, the early development of a primordial follicle to the preantral stage is characterized by enlargement of the oocyte, proliferation of the granulosa cells and development of a theca cell layer (Peters, 1979). The majority of growing follicles will become atretic at this stage (Brand & de Jong, 1973). The further development of surviving preantral follicles involves recruitment, selection and dominance (Goodman & Hodgen, 1983). Graafian follicles, formed following follicular recruitment, are characterized by a follicular antrum filled with follicular fluid surrounded by granulosa and theca cell layers. The oocyte is surrounded by the corona radiata and

attached to the antrum wall by the cumulus oophorus (Erickson, 1986). In sheep it has been estimated to take 6 months for a primordial follicle to develop into a large dominant follicle (Cahill & Mauléon, 1980) but 22 days for the early antral follicle to develop to the preovulatory stage (Scaramuzzi, Turnbull & Nancarrow, 1980). If luteal regression occurs, the dominant follicle continues to develop to preovulatory size. Ovulation occurs with the release of the oocyte and the follicular remnants hypertrophy to form the CL (Johnson & Everitt, 1988).

The development of real-time B-mode ultrasound has enabled the growth pattern of CL, and individual follicles larger than 5 mm in diameter, to be followed (Pierson & Ginther, 1984, 1986, 1987a,b,c, 1988; Quirk & Fortune, 1986). Follicle growth and development in the bovine oestrous cycle occurs in waves (Savio, Keenan, Boland & Roche, 1988; Sirois & Fortune, 1988; Knopf, Kastelic, Schallenberger & Ginther, 1989). Each wave is characterized by the simultaneous emergence of a group of growing follicles, one of which is selected to become dominant, while the other cohort follicles become atretic and regress. The dominant follicle reaches a diameter of 10-15 mm and remains at this size for 2-3 days before regression or further development to the preovulatory stage. Three waves of follicular development were observed in the majority of bovine oestrous cycles (Savio *et al.*, 1988; Sirois & Fortune, 1988) with the first, second and third waves starting on approximately Days 2, 10 and 16 of the cycle respectively, although other workers identified a 2-wave pattern with the first and second waves starting on Days 1 and 10. Since animals with a 2-wave pattern tended to have shorter cycles (Sirois & Fortune, 1988; Ginther, Knopf & Kastelic, 1989a; Knopf *et al.*, 1989) and since wave patterns are present throughout pregnancy (Ginther, Knopf & Kastelic, 1989b; Driancourt, Thatcher, Terqui & Andrieu, 1991), it is likely

that the number of waves per cycle is determined by the length of the cycle rather than being an intrinsic characteristic of the cycle.

Ireland, Murphree & Coulson (1980) described 4 readily-identifiable changes in gross appearance of CL during the bovine oestrous cycle. In stage I (Days 1-4) the CL is 0.5-1.5 cm in diameter, red and not covered by epithelium and has a raised apical ovulation point. In stage II (Days 5-10) the CL achieves its maximum size of 1.6-2.0 cm in diameter, the apex is epithelialised and is red or brown, while the remainder of the CL is orange. In stage III (Days 11-17) the apex of the CL is tan/orange and covered by a vascular network. The centre may be occupied by a cavity which is usually small, but which can range from 0.4-1.0 cm in diameter, and contains a yellow fluid (Arthur *et al.*, 1982). Following the onset of luteolysis in stage IV (Days 18-20) the CL undergoes rapid reduction in size and a change in colour through light-yellow to white.

1.5. Formation and structure of the corpus luteum

The Graafian follicle is the progenitor of the CL; therefore extrafollicular and intrafollicular events affect subsequent luteal function.

1.5.1. Follicle maturation, ovulation and luteinization

Much of the literature regarding the developmental changes in follicular function has been derived from studies in rats and mice. For extensive descriptions of follicular morphology, the reader is referred to Rajakowski (1960) and Mossman and Duke (1973). The hallmark of a follicle that is ready to ovulate and form a CL is the acquisition of LH receptors by the granulosa cells. Initially the granulosa cell layer of the follicle contains only receptors for FSH (Richards, 1980), whereas theca cells have receptors for LH but not for FSH. Stimulation of the granulosa cells by FSH induces

cellular proliferation (Rao, Midgley & Richards, 1978), the development of aromatase activity (Dorrington, Moon & Armstrong, 1975), and the formation of functional LH receptors in the granulosa cells (Zelevnik, Midgley & Reichert, 1974). LH stimulates the theca cells to produce androgens that can be aromatized by the granulosa cells (Fortune & Armstrong, 1977). Thus oestrogen is synthesized through the combined activities of both theca and granulosa in the presence of both LH and FSH. Oestradiol synergises with FSH in the formation of FSH and LH receptors. Once the granulosa cells have acquired LH receptors, LH stimulates synthesis of oestradiol and promotes further development of the follicle, acquisition of additional LH receptors by the granulosa cells and functional and morphological maturation of the theca cells (Ireland & Richards, 1978, Bogovich, Richards & Reichert, 1981). When one of the growing follicles emerges as the large dominant follicle it is characterized by its high oestradiol production and large number of granulosa LH receptors (Staigmiller, England, Webb, Short & Bellows, 1982). Porcine granulosa cells from the mature follicle rendered responsive to LH have the capacity to undergo luteinization (the series of morphological and biochemical changes in the cells of the theca interna and membrana granulosa of the preovulatory follicle) and synthesize progesterone only after exposure to elevated concentrations of LH (Channing, Brinkley & Young, 1980a). In ewes morphological signs of luteinization were evident 6-12 hours after ovulation (McClellan, Diekman, Abel & Niswender, 1975). The ability of LH to induce luteinization is related to its ability to increase intracellular concentrations of cyclic adenosine 3',5'-monophosphate (cAMP) (Richards, Jonassen, Rolfes, Kersey & Reichert, 1979). If the follicle is destined to ovulate, declining progesterone concentrations coincident with luteolysis allow increased LH secretion (Ireland & Roche, 1982), driving the final stages of maturation and ovulation.

The preovulatory gonadotrophin surge stimulates the follicle to ovulate and form a CL. The process of ovulation is reviewed by Channing, Schaerf, Anderson & Tsafiriri (1980b). However, ovulation *per se* does not guarantee normal luteal development and function, and other extrafollicular and intrafollicular endocrine and cellular changes can affect the function of the subsequent CL. Currently it is unclear whether follicular events at the time of follicular recruitment or events during the preovulatory period are most directly associated with subsequent luteal function (Smith, 1986). In both cows and ewes a decrease in preovulatory FSH, but not LH, concentrations was detected prior to a short versus a normal length oestrous cycle (Ramirez-Godinez, Kiracofe, Schalles & Niswender, 1982; McNeilly, 1984). Pulsatile injections of GnRH (2 µg every 2 hours) for 72 hours prior to a GnRH challenge (50 µg) in post-partum anoestrous dairy cows resulted in oestrous cycles of normal length, whereas a GnRH challenge preceded by saline injections resulted in short cycles (Mollett, Smith & Garverick, 1983). In addition to FSH, progesterone contributes to the preovulatory endocrine environment. Injection of anoestrous cows with GnRH resulted in formation of short-lived CL unless the animals were pretreated with a progestagen (Troxel & Kesler, 1983). In cycling cows elevated plasma progesterone concentrations during the luteal phase were followed by reduced plasma progesterone concentrations during the subsequent oestrous cycle (Milvae, Murphy & Hansel, 1984). It is unclear whether the effect of progesterone priming on subsequent luteal function was due to the modulation of preovulatory gonadotrophin secretion or to a direct effect on the preovulatory follicle. As described above, intrafollicular gonadotrophin and oestradiol concentrations are important in the preparation of a preovulatory follicle for luteinization and normal luteal function (McNatty, 1979). Premature ovulation by intrafollicular injections of FSH or LH caused luteal

insufficiency in the ewe (Murdoch, de Silva & Dunn, 1983). Granulosa cells harvested from Graafian follicles containing elevated concentrations of FSH, LH and oestradiol-17 β , produced the most progesterone *in vitro* (McNatty & Sawers, 1975).

The formation of the CL is initiated by a series of morphological and biochemical changes in the cells of the theca interna and membrana granulosa, termed luteinization. Luteinization marks the termination of replication for most granulosa cells (Rao *et al.*, 1978) and their differentiation into cells with new characteristics that include a striking hypertrophy and increase in the enzyme systems and organelles that typify steroid-producing cells (Priedkalns & Weber, 1968; Enders, 1973). As a result of the breakdown of the blood-follicle barrier between the granulosa and the theca, there is invasion by endothelial cells and the co-mingling of theca cells and granulosa cells, with the establishment of new cell associations (Bloom & Fawcett, 1975). During the preovulatory gonadotrophin surge, oestrogen secretion begins to decrease precipitously (Webb, England & Fitzpatrick, 1981a) and there is a dramatic shift in steroid production from androgens and oestrogens towards progesterone apparently triggered through the action of LH (Keyes, Gadsby, Yuh & Bill, 1983). Associated with these preovulatory changes in steroidogenesis there is a marked decrease in receptors for LH, loss of the FSH receptor (Rao, Richards, Midgley & Reichert, 1977), and the loss of aromatase activity in ruminants (Henderson & Moon, 1979).

1.5.2. Angiogenesis

Development of the preovulatory follicle is linked to the acquisition of a rich vascular supply in the surrounding thecal area of the follicle, permitting preferential uptake of gonadotrophins (Di Zerega & Hodgen,

1980). The Graafian follicle has a basal lamina separating the avascular interior of the follicle from the profuse network of blood vessels that lie in the theca interna. At, or prior to, ovulation the basal lamina, which is composed of type IV collagen, laminin and fibronectin, breaks down, allowing rapid ingrowth of capillaries from the thecal capillary wreath (Koos & LeMaire, 1983). In cattle the granulosa cell layer is invaded by capillaries within several hours of ovulation; however the luteal capillary network is not complete until 9 days after oestrus (Koos & LeMaire, 1983). As a result of the proliferation of endothelial cells, a profuse network of new sinusoidal vessels is formed endowing the CL with an exceptionally high blood flow (Keyes & Wiltbank, 1988). Thus angiogenesis is a prominent feature of luteinization. Blood flow to the luteal ovary of the sheep increases from less than 1 ml/min to 3-7 ml/min as the CL develops and is maintained (Niswender, Reimers, Dickman & Nett, 1976), and in the cow, from 1.4 ml/min at oestrus to 2.7 ml/min during the luteal phase of the oestrous cycle (Ford & Chenault, 1981). To explain the rapid attraction of such an extensive vascular supply Jakob, Jentzsch, Mauersberger & Oehme (1977) first demonstrated angiogenic activity in the bovine CL, and Gospodarowicz & Thakral (1978) described the existence of a diffusible substance emanating from corneal transplants of 4-day old luteal tissue, which induced capillary migration and neovascularization of the graft; non-luteinizing follicles failed to induce neovascularization. While the initial stimulus is hypothesized to be the increased demand for oxygen of the luteal cells (Gospodarowicz & Thakral, 1978), one factor involved in capillary proliferation is a mitogenic factor, subsequently purified and shown to be related to fibroblast growth factor (Gospodarowicz, Cheng, Lui, Baird, Esch & Bohlen, 1985). Prostacyclin (PGI₂) is a potent vasodilator (Moncada & Vane, 1979) and may be an important regulator of blood flow assisting in angiogenesis during luteal development (Smith, 1986); prostacyclin may stimulate progesterone secretion by a direct

effect on luteal cells, or indirectly by increasing luteal blood flow, so further increasing the amount of LH reaching the CL.

1.5.3. Ontogeny of luteal cells

Many studies in cattle (McNutt, 1924; Gier & Marion, 1961; Donaldson & Hansel, 1965a; Friedkalns & Weber, 1968; Alila & Hansel, 1984), sheep (McClellan *et al.*, 1975; O'Shea, Cran & Hay, 1980), sows (Corner, 1919) and guinea pigs (Loeb, 1906) provide evidence that both the granulosa and theca layers contribute cells to the developing CL. This conclusion is based on sequential observations of light and electron microscope structure together with the use of 'markers'. Glycogen bodies, involving whorled arrangements of smooth membranes, are a characteristic feature of granulosa cells in sheep preovulatory follicles and persist for 24-48 hours after ovulation, but are not seen in thecal cells, so form a convenient ultrastructural marker of granulosa-derived cells in the early post-ovulatory period (O'Shea *et al.*, 1980). In sheep alkaline phosphatase is confined to the theca in preovulatory follicles; use of this marker has clearly demonstrated the incorporation of theca cells in the developing CL and supported the concept of a thecal origin for the small luteal cells (O'Shea *et al.*, 1980). Monoclonal antibodies raised against bovine granulosa cell surface antigens have pointed to the granulosa as the origin of large luteal cells (Alila & Hansel, 1984) and similarly antibodies against bovine theca cell surface antigens have indicated the theca as a source of at least a large proportion of small luteal cells in the cow. Finally, numbers of granulosa cells in preovulatory sheep follicles are similar to numbers of large luteal cells per CL (Rodgers, O'Shea & Bruce, 1984) which would be expected in view of evidence that granulosa-derived cells undergo little, if any, post-ovulatory mitosis (McClellan *et al.*, 1975).

The precise nature of the contributions of granulosa and theca cells to the developing and established CL, and the question as to whether large luteal and small luteal cells persist as discrete and closed populations throughout the lifespan of the CL, are more controversial. The conclusion from a study in sheep (O'Shea, Rodgers & Wright, 1986) was that once formed, the cells of the CL are relatively static for the remainder of the oestrous cycle with very little mitosis, cell death or redifferentiation occurring. However, Farin, Moeller, Sawyer, Gamboni & Niswender (1986) observed an increase in the number of small luteal cells throughout the oestrous cycle, and McClellan *et al.* (1975) observed some mitoses in thecal cells in the early luteal phase, though nearly 90% of cell division occurred in fibroblasts and endothelial cells. However Parry, Willcox & Thorburn (1980) measured an increase in total luteal cells by morphometry in the cow between Days 6 and 13. Furthermore using staining for 3 β -hydroxysteroid dehydrogenase activity, an enzyme which converts pregnenolone to progesterone, as a marker of steroidogenic cells, Niswender, Schwall, Fitz, Farin & Sawyer (1985) detected a several-fold increase between Days 4 and 8 in the number of luteal cells in cycling ewes.

Several lines of evidence suggest that small luteal cells may be able to differentiate into large luteal cells. Friedkalns & Weber (1968) recognized the presence of luteal cells of intermediate structure, and Parry *et al.* (1980) were subsequently unable to distinguish large luteal and small luteal cells as separate cell types in the bovine CL. However Fields, Dubois & Fields (1985) failed to observe cells of intermediate structure. Fitz, Sawyer & Niswender (1981) found an increase in large luteal cell number and a decrease in small luteal cell number in the later stages of the oestrous cycle of the sheep, suggesting the possibility of transformation of some small luteal cells to large luteal cells. Additionally Niswender *et al.* (1985) demonstrated a rise in

the number of large luteal cells and a corresponding decrease in small luteal cells in the cyclic sheep CL in response to luteotrophic stimulation by human chorionic gonadotrophin (hCG). However these observations were based on cells obtained by enzymic dispersion; a subsequent study based on morphometry failed to show any rise in large luteal cell number (O'Shea *et al.*, 1986). In a study by Cran (1983) luteinization of follicular cysts formed in response to pregnant mare serum gonadotrophin (PMSG), in which granulosa cells had largely degenerated, was accompanied by the formation of cells whose ultrastructure closely resembled that of large luteal cells; the strongest evidence in sheep that theca-derived cells may be able to differentiate into large luteal cells. With the use of specific monoclonal antibodies Alila & Hansel (1984) observed that the number of large luteal cells binding a granulosa-specific antibody declined progressively throughout the oestrous cycle, while the number binding a theca-specific antibody increased. The majority of small luteal cells bound the theca-specific antibody, and no small luteal cells bound the granulosa-specific antibody after Day 6 of the cycle, adding strength to the case for transformation of theca-derived cells to large luteal cells. No cells were bound by granulosa-specific antibody after 100 days of pregnancy indicating that the granulosa-derived large luteal cell line has a limited life span and disappears during gestation, or that the granulosa cell-specific antigen was lost. In contrast, cells of thecal origin persisted throughout gestation. The existence of a population of stem cells which give rise to small steroidogenic luteal cells which in turn develop into large luteal cells is suggested by Niswender *et al.* (1985) as a mechanism to explain the above observations. Donaldson & Hansel (1965a) previously suggested that LH may regulate the differentiation of small to large luteal cells in cattle.

Thus the case for cellular transformation remains unresolved. Both granulosa and theca cells are extragonadal in origin (Byskov, 1986). The granulosa cells are derived from the extraembryonic endoderm and the theca cells from the mesoderm during ovarian histogenesis. At the time of folliculogenesis granulosa and theca cells are structurally and functionally distinct cell types and are separated by a complete basal lamina until close to the time of ovulation. O'Shea (1987) argues that subsequent convergent differentiation to a common, new type of specialized cell, the large luteal cell, would appear intrinsically surprising.

1.5.4. Structure of the corpus luteum

The CL in ruminants, as in other mammals, contains specific hormone producing (luteal) cells, endothelial cells, pericytes, smooth muscle cells, fibrocytes, macrophages, leucocytes and occasional plasma cells (Rodgers *et al.*, 1984).

1.5.4.1. The luteal cells

In several groups of mammals, including perissodactyls (eg. horses), cetaceans (eg. whales, dolphins) and artiodactyls (eg. pigs, ruminants), cellular heterogeneity is also seen within the population of steroidogenic luteal cells. In all of these groups two distinct populations of luteal cells have been recognized histologically (Corner, 1919; Mossman & Duke, 1973), and variously termed large and small luteal cells, or granulosa and theca luteal (or lutein) cells on account of their putative origins. Furthermore, with the use of specific monoclonal antibodies Alila & Hansel (1984) observed heterogeneity within the large luteal cell population. Criteria for identification of these populations based on cell diameter after tissue dispersion have varied substantially between studies. In the CL of the

pregnant cow Chegini, Ramani & Rao (1984) regarded cells $>18\text{ }\mu\text{m}$ in diameter as large luteal cells, whereas Weber, Fields, Romrell, Tumwasorn, Ball, Drost & Fields (1987) used $23\text{ }\mu\text{m}$ diameter as their criterion. In the CL of the cyclic cow Koos & Hansel (1981) used $25\text{ }\mu\text{m}$ diameter and Rodgers, Rodgers, Waterman & Simpson (1986a) used $26\text{ }\mu\text{m}$ as the smallest diameter for the large luteal cell population. On the basis of counts of dispersed cell populations Hansel, Alila, Dowd & Yang (1987) estimated that small bovine luteal cells outnumbered large luteal cells by a ratio of 20:1-40:1, whereas Weber *et al.* (1987) reported a ratio of only 10.2:1. In sheep it has been found that dispersion of luteal tissue may result in selective loss of certain cell types such that counts of dispersed populations may not accurately reflect the tissue populations (Rodgers *et al.*, 1984). Similar observations in the cow (O'Shea, Rodgers & D'Occhio, 1989) suggest that there is a preferential loss of large luteal cells. Ultrastructural morphometry, in which non-measurable losses do not arise, suggests that the cyclic CL of the cow contains 392.4×10^6 small luteal cells and 51.5×10^6 large luteal cells, the data indicating a ratio of 7.6:1 (O'Shea *et al.*, 1989). Of the total of 393.4×10^3 cells per mm^3 of luteal tissue, large luteal cells made up only 3.5% and small luteal cells 26.7% of cell number. Luteal cells therefore represent a minority of the total cells per CL. Endothelial cells and pericytes, at 52.3% were the most numerous cell type. A similar observation has been made in the ovine CL (Rodgers *et al.*, 1984), and attests to the very high vascularity of luteal tissue. Estimates of the percentage composition (volume density) of mature functional CL, based on point count measurements, showed that small and large luteal cells occupied 67.9% (O'Shea *et al.*, 1989), and $>70\%$ (Parry *et al.*, 1980), of luteal tissue. Converting volumes to a spherical shape O'Shea *et al.* (1989) provided estimates of mean diameter of $38.4\text{ }\mu\text{m}$ and $17.2\text{ }\mu\text{m}$ for large and small luteal cells respectively. The cytoplasmic:nuclear ratio of large luteal cells was ≈ 4.5 -fold greater than that of small luteal cells (O'Shea *et al.*, 1989). Granulosa

lutein cells are undoubtedly the largest strictly-endocrine cells in the body (Enders, 1973).

Ultrastructural features of small and large luteal cells of cyclic and pregnant cows are described in the studies of Priedkalns & Weber (1968) and Fields *et al.* (1985) respectively. In addition to differences in size and shape, O'Shea (1987) considers that large luteal cells differ from small luteal cells primarily in possessing large numbers of secretory granules, an observation further confirmed by O'Shea, Rodgers, McCoy & D'Occhio (1990). Although both types of luteal cell possess fine structural features typical of steroid-secreting cells, the large luteal cell also has characteristics normally found in cells specialized for the secretion of polypeptides and proteins (Niswender *et al.*, 1985). Thus large luteal cells not only have numerous mitochondria and an abundance of smooth endoplasmic reticulum, consistent with a steroid-secreting function, but they also have an elaborate and extensive endomembrane system (i.e. numerous Golgi complexes, rough endoplasmic reticulum and secretory granules) consistent with a protein-secreting function. Details of ultrastructural features from other studies are inconsistent. In the cyclic Day 11-13 CL of the cow Koos & Hansel (1981) observed that small luteal cells had acentric, deeply indented, cup-shaped nuclei with heterochromatin lining the nuclear envelope, a relatively smooth surface except for some microvilli, both rough and smooth endoplasmic reticulum, pleomorphic mitochondria with tubular cristae arranged in an arc opposite the nucleus and a central region that usually contained a pair of centrioles and a large Golgi complex. Large luteal cells had central round nuclei with dispersed chromatin and a distinct nucleolus, two types of mitochondria, extensive smooth endoplasmic reticulum, and a highly convoluted cell surface and small (0.3 μ m) electron-dense granules. Singh (1975) identified stacks of rough endoplasmic reticulum and whorls of

smooth endoplasmic reticulum as features of large (granulosa lutein) cells in pregnant cows. However other studies have reported stacks of rough endoplasmic reticulum, whorls of smooth endoplasmic reticulum and the presence of "crystalline-like" inclusions in some of the mitochondria to be features of small luteal cells which are not observed in large luteal cells (Fields *et al.*, 1985). Chegini *et al.* (1984) considered small and large luteal cells of CL obtained from pregnant animals to be morphologically similar, but quantitatively there were more cytoplasmic organelles in large compared to small luteal cells. Parry *et al.* (1980) were unable to confirm the existence of two distinct cell types in the cyclic CL by electron microscopy, regarding all the luteal cells as "part of the same population". At least three types of granules were present in mid-luteal cells (Parry *et al.*, 1980): microperoxisomes, primary lysosomes and secretory granules. Only the last appear to be exocytosed. Electron-dense secretory granules were 0.2-0.4 μm in diameter, enclosed by a single membrane, usually spherical, and distributed in clusters throughout the cytoplasm of large luteal cells, and observed in the extracellular space, suggesting they had been secreted. Microperoxisomes (0.15-0.25 μm diameter), were indistinguishable from secretory granules morphologically, but identified by their peroxidase staining and found adjacent to lipid droplets, suggesting an involvement in steroidogenesis. Lysosomes, a morphologically heterogeneous group of cytoplasmic particles, which by virtue of their content of acid hydrolases, act as an intracellular digestive system, were observed at all stages of the ovine cycle (Gemmell, Stacy & Thorburn, 1976). Lipid droplets, present in small luteal cells, are virtually absent in large luteal cells (Niswender & Nett, 1988).

Changes in the proportion of organelles throughout the oestrous cycle reflect changes in luteal function. Cytoplasmic analysis of luteal cells on five

different days of the oestrous cycle, (Parry *et al.*, 1980), revealed an increase in concentration of mitochondria at mid-cycle correlating with the expected requirement for greater amounts of cholesterol side-chain cleavage activity to accompany increased progesterone synthesis. The concentration of cholesterol-containing lipid droplets varied inversely with the rate of progesterone synthesis, in agreement with their projected role as stores of steroid precursors. Numbers of droplets fell as the rate of secretion of progesterone by luteal cells increased, but rose at the end of the cycle indicating a failure of steroid biosynthesis: the cells were able to accumulate lipid but were unable to achieve the conversion of lipid to steroid hormone. The protein-synthesizing compartment, consisting of granular endoplasmic reticulum plus polysomes and/or ribosomes, increased steadily from Day 6 to Day 17. Agranular endoplasmic reticulum and groundplasm (the amorphous ground substance containing tissue fluid in which cells and extracellular fibres are embedded) were the most abundant cytoplasmic components, as expected in a steroidogenically active tissue. Fields *et al.* (1985) demonstrated that morphological changes occurring in bovine luteal cells during pregnancy were very similar to those reported for the non-pregnant cow.

Biochemically, small and large luteal cells are similar (Chegini *et al.*, 1984). Measurement of the activities of enzymes that are directly involved in progesterone biosynthesis (HMG-CoA reductase and 3 β -hydroxysteroid dehydrogenase) or indirectly involved by providing reducing equivalents of energy (glucose-6-phosphate dehydrogenase, NADH cytochrome c reductase and cytochrome c oxidase) in the bovine CL of pregnancy, revealed two differences. The activities of 5'-nucleotidase and NADH cytochrome c reductase were significantly lower in large as compared to small luteal cells. Rodgers, Waterman & Simpson (1986b) found cholesterol side-chain cleavage cytochrome P-450, an inner mitochondrial enzyme which catalyzes the rate-

limiting step in the conversion of cholesterol to progesterone, in both small and large luteal cells of the cyclic CL, but concentrations were greater in the large than in the small luteal cell, reflecting the greater numbers of mitochondria per cell.

In the ewe the small luteal cells contained the majority of the LH receptors (33260/small cell compared to 3074/large cell) (Fitz, Mayan, Sawyer & Niswender, 1982). In a more recent study the large luteal cells from Day 10 and Day 15 ovine CL had as many receptors for LH/hCG on a per cell basis as did small luteal cells (Harrison, Kenny & Niswender, 1987). The discrepancy may be due to the use of luteal cells from spontaneously ovulating ewes in the latter study compared to the CL of animals induced to superovulate with PMSG. The majority of the binding sites for prostaglandin E₂ (PGE₂) and prostaglandin F_{2α} (PGF_{2α}) were found on the large cells (10955 and 68143 sites respectively) compared to 904 sites for PGE₂/small cell and 2115 sites for PGF_{2α}/small cell (Fitz *et al.*, 1982). Large and small luteal cells of the sheep have high and low affinity receptors for PGF_{2α} respectively (Balapure, Caicedo, Kawada, Watt, Rexroad & Fitz, 1989) and thus significant occupancy of the lower affinity sites on small luteal cells would require higher concentrations of PGF_{2α}.

Intercellular junctions are common in follicles, particularly gap and adherens-type junctions (Priedkalns & Weber, 1968; McClellan *et al.*, 1975) in the granulosa^{ous}. Gap junctions allow the passage of substances of very low molecular weight (MW) (e.g. cAMP) from one cell to another, and as such are a means by which cell-cell communication can occur. Adherens-type junctions serve to bind cells together and probably have little role in cell-cell communication (Rodgers, 1990). On ovulation, in sheep, the number of gap junctions between the granulosa cells declines (McClellan *et al.*, 1975), enabling the theca cells to penetrate between them as the area occupied by

the granulosa cells is vascularized. Gap junctions are rare in mature CL of sheep and cattle (McClellan *et al.*, 1975). Septate junctions have been observed between small theca-derived cells of bovine CL (Rodgers *et al.*, 1986a). These areas of close abutment with associated adherens-type junctions are characterized by parallel segments of apposing membranes separated by a gap of up to 10 nm.

1.5.4.2. The vasculature

In 1898 Prenant hypothesized that the CL was an endocrine gland because of "its abundant vascularity, a sign by which the histologist characterizes a gland of internal secretion pouring its products into the internal environment of the organism via the blood", an impression subsequently confirmed by direct measurement. Luteal blood flow (per unit of tissue) is much greater than blood flow to other organs, and accounts for approximately 90% of total ovarian blood flow during the mid-luteal phase (Bruce & Moor, 1976); luteal blood flow is positively correlated to mean arterial pressure (Wiltbank, Gallagher, Christensen, Brabec & Keyes, 1990). Vascular resistance in the ovarian stroma, as in most tissues, is acutely regulated by dilation or constriction of intra-tissue arterioles; in contrast vascular resistance within the CL is a relatively invariable parameter, fixed at a low level by the morphological characteristics of the luteal vasculature: large sinusoidal capillaries lacking smooth muscle (Wiltbank *et al.*, 1990). Therefore the CL operates on a linear (maximally "vasodilated") pressure-flow curve, does not actively regulate intra-tissue blood flow, and is subject to acute regulation of perfusion only through changes in extraluteal vessels (Wiltbank *et al.*, 1990). Further support for this extraluteal regulation of blood flow is provided by the apparent lack of innervation to the CL (Unsicker, 1974), the failure of luteotrophic hormones to stimulate luteal blood flow and the failure of withdrawal of the luteotrophic hormone

oestradiol-17 β to diminish luteal blood flow in rabbits (Keyes & Wiltbank, 1988).

In the cow a significant portion (20-40%) of the blood flow to the ovary containing a functional CL is supplied by the ipsilateral uterine artery through an anastomosis with the ovarian artery (Ford & Chenault, 1981). However the quantity of blood and the direction of flow through this anastomosis ^{sense?} changed through the oestrous cycle. As a result of the high vascularity of luteal tissue, the large luteal cells, which comprise the bulk of the mid-luteal CL, were always close to capillaries. The CL can account for as much as 90% of the total blood flow to the CL-containing ovary in the ewe (Ellinwood, Nett & Niswender, 1978), ensuring the supply of essential nutrients, luteotrophic hormones and cholesterol in a metabolically active tissue. Furthermore, progesterone has been shown to be a competitive inhibitor of the enzyme 3 β -hydroxysteroid dehydrogenase/ Δ^5, Δ^4 -isomerase, which converts pregnenolone to progesterone, and thus rapid product removal is important to ensure a high rate of progesterone secretion (Ellinwood *et al.*, 1978). During the period of maximum progesterone secretion, vascular elements account for approximately 11% of luteal volume in the Day 9-10 ovine CL (Nett, McClellan & Niswender, 1976) and 7% of luteal volume in the Day 13 bovine CL (Parry *et al.*, 1980).

1.6. Function of the corpus luteum

1.6.1. Steroidogenesis

An intrinsic property of the luteal cell is the capacity for *de novo* steroidogenesis (Savard, 1973), which is inherited from its progenitors, the granulosa cell and the theca cell (Enders, 1973). Although there is some evidence that macrophages may serve a specific stimulatory function in relation to progesterone synthesis (Kirsch, Vogel & Flickinger, 1983), the

non-luteal cell population is presumed to be primarily concerned with their conventional ancillary roles. The preovulatory LH surge stimulates luteinization (Keyes *et al.*, 1983), greatly expanding the capacity for progesterone synthesis. In some species such as the cow (Henderson & Moon, 1979), aromatase activity disappears within a few days of ovulation; in other species such as the rat (Elbaum & Keyes, 1976), the CL retains aromatase activity and produces oestrogen. Steroidogenesis in luteal tissue is less complex than in other steroidogenic tissues, since the major product, progesterone, is formed early in the steroidogenic pathway. Cholesterol is the initial substrate for luteal steroidogenesis. It may be either extracted from circulating low density lipoprotein (LDL) or (in some species) high density lipoprotein (HDL) produced by the liver, or released from intracellular stores of cholesterol esters, or synthesized *de novo* from acetate. Although all three sources of cholesterol have been implicated in the steroidogenic response of luteal cells to LH, there are substantial quantities of cholesterol (both free and esterified) already present within luteal cells (Niswender & Nett, 1988). The steroidogenic luteal cell contains LDL (or HDL) receptors that are involved in the uptake of lipoprotein by the cell (Niswender & Nett, 1988). The LDL-LDL receptor complex is internalized by endocytosis. The endocytotic vesicles then fuse with lysosomes and the cholesterol is liberated. The free cholesterol leaves the lysosome and is either esterified and stored as lipid droplets, used for steroid biosynthesis, or used in the formation of the membrane constituents of the cell. For biosynthesis of progesterone, cholesterol is transported to the mitochondria where it is converted to pregnenolone by side-chain cleavage enzyme. The pregnenolone is converted to progesterone by 3β -hydroxysteroid dehydrogenase/ Δ^5, Δ^4 -isomerase in the smooth endoplasmic reticulum, and the progesterone is secreted. The luteal cell has a limited capacity to store progesterone (Niswender & Nett, 1988).

Evidence from several studies of cattle (Ursely & Leymarie, 1979; Koos & Hansel, 1981), sheep (Fitz *et al.*, 1982; Rodgers, O'Shea & Findlay, 1983a), and pigs (Lemon & Loir, 1977), have indicated a marked difference in steroidogenic response to LH by large luteal and small luteal cells *in vitro*. Basal progesterone secretion was lower in small luteal cells relative to large luteal cells; however small luteal cells from bovine and ovine CL were extremely responsive to added LH, while large luteal cells from both species were relatively unresponsive to LH (Alila, Dowd, Corradino, Harris & Hansel, 1988a; Farin, Sawyer & Niswender, 1989a). Specifically, basal secretion of progesterone by large bovine luteal cells is 20 times greater than small luteal cells. However small luteal cells were 6 times more responsive to LH than large luteal cells (Koos & Hansel, 1981). Evidence from studies in the cow (Hansel, Alila, Dowd & Milvae, 1991) and ewe (Harrison *et al.*, 1987), support the hypothesis that during maintenance of the CL, the large and small luteal cells synergize to promote the production of progesterone, and during regression of the CL this synergism is absent.

The LH response by small luteal cells is mediated primarily by cAMP, intracellular concentrations of which are elevated in response to LH (Hoyer, Fitz & Niswender, 1984). Dibutyryl cAMP (Fitz *et al.*, 1982; Rodgers *et al.*, 1983a), or activators of adenylate cyclase (Hoyer *et al.*, 1984), also stimulate progesterone production by these cells. Progesterone synthesis by small luteal cells is also stimulated by hCG, although quantitative aspects of the response differ from those of LH (Niswender *et al.*, 1985). An exception to the response of small luteal cells to LH is found in cows during the later stages of pregnancy (Chegini *et al.*, 1984; Weber, Roberts, Romrell & Fields, 1984), and may represent a regressionary change.

Nishizuka (1986) described two classes of cells having either "monodirectional" or "bidirectional" control systems, in which two classes of receptors (those that are cAMP-generating and those involving Ca^{2+} mobilization, protein kinase C activation, arachidonate release and cyclic guanosine monophosphate (cGMP) formation) either potentiate one another or have opposing effects. Pituitary cells are examples of monodirectional cells in which protein kinase C appears to potentiate cAMP production. Small luteal cells behave as monodirectional cells, insofar as both second messenger systems stimulate progesterone synthesis. Time course studies have revealed that LH-induced increases in inositol 1,4,5-trisphosphate (IP_3) and cAMP occur simultaneously and precede the increases in progesterone secretion (Davis, Alila, West, Corradino, Weakland & Hansel, 1989). Augmentation of receptor-linked adenylate cyclase activity was observed following pretreatment of luteal cells with the phorbol ester, phorbol-12-myristate-13-acetate (PMA), suggesting that at the membrane level the LH receptor-linked adenylate cyclase and protein kinase C signalling systems can interact (Budnik & Mukhopadhyay, 1987). However Hansel *et al.* (1987) found no evidence that the protein kinase C system potentiates cAMP production in small luteal cells. In contrast in bidirectional cells, the two classes of receptor counteract each other, and protein kinase C inhibits and desensitizes the adenylate cyclase system. Moreover, a major function of protein kinase C appears to be the down-regulation of cell surface receptors; this action of protein kinase C extends to the receptors of other signalling systems (Nishizuka, 1986). Ovarian granulosa cells are examples of bidirectional cells. The large luteal cells may behave as bidirectional cells, although Hansel *et al.* (1987) were unable to demonstrate a clear inhibitory effect of protein kinase C activation on LH-stimulated progesterone synthesis. However LH receptors may already have been down-regulated in

these mid-cycle large luteal cells, perhaps as a result of the activation of the protein kinase C pathway.

Many lines of evidence support a role for calcium in the control of steroidogenesis. Elevated intracellular calcium inhibited (Hansel & Dowd, 1986) or had no effect (Hansel *et al.*, 1987) on LH-stimulated cAMP-mediated progesterone synthesis in small luteal cells. Mobilization of intracellular calcium is an integral part of the polyphosphoinositol protein kinase C second messenger system (Hansel *et al.*, 1991). Transmembrane signalling of second messengers by LH and PGF₂ α in mixed populations of bovine luteal cells involves increases in intracellular calcium concentration ([Ca]_i) (Davis, Weakland, Farese & West, 1987a; Davis *et al.*, 1988), but the magnitude and profile of intracellular calcium changes induced by LH, differ greatly in small and large luteal cells (Alila, Corradino & Hansel, 1989). Small luteal cells contain relatively low resting [Ca]_i, produce basal progesterone in the absence of calcium in the medium, but progesterone synthesis stimulated by LH, PGE₂, 8-bromo-cAMP and PGF₂ α requires calcium. Production of cAMP is not altered by low extracellular calcium: inhibitory effects of calcium ion removal are exerted distal to cAMP (Hansel *et al.*, 1991). Low concentrations of LH increase [Ca]_i and progesterone synthesis 6-fold in small luteal cells (Alila *et al.*, 1989). Large luteal cells contain high resting [Ca]_i and require calcium for basal, LH- and forskolin-stimulated progesterone production (Alila *et al.*, 1988a; Alila *et al.*, 1989). 8-bromo-cAMP-stimulated progesterone production does not require calcium: inhibitory effects of calcium ion removal are exerted proximal to cAMP generation (Hansel *et al.*, 1991). High concentrations of LH increase [Ca]_i and progesterone synthesis 1.5-2 fold in large luteal cells (Alila *et al.*, 1989). Following LH the increase in [Ca]_i occurs in two phases in the small luteal cell; the first phase is due to mobilization of intracellular calcium by IP₃ and triggering of calcium influx by inositol

1,3,4,5-tetrakisphosphate (IP_4); the second phase is a result of influx of extracellular calcium, as indicated by the fact that it disappears after the addition of EGTA, a calcium chelator, to the medium (Hansel *et al.*, 1991). In contrast only a single phase of intracellular calcium increase was observed in LH-treated large luteal cells and was abolished by EGTA, indicating that the LH-induced rise in $[Ca]_i$ in these cells is entirely dependent on an IP_3 -independent extracellular ion influx (Hansel *et al.*, 1991).

There are several differences in the control of steroidogenesis between cattle and sheep. Large luteal cells from both species are relatively insensitive to LH (Alila *et al.*, 1988a; Farin *et al.*, 1989a). However large bovine luteal cells, in contrast to large ovine luteal cells, respond to high (≥ 100 ng/ml) concentrations of LH and to forskolin and 8-bromo-cAMP with an approximate doubling of progesterone synthesis. Addition of $PGF_{2\alpha}$ to dispersed bovine luteal cells resulted in increased progesterone synthesis (Hixon & Hansel, 1979): this stimulation has since been shown to be limited to the small theca-derived cells (Alila *et al.*, 1988a). $PGF_{2\alpha}$ does not stimulate progesterone production in ovine small luteal cells (Wiltbank, Diskin & Niswender, 1991). However an inhibitory effect of $PGF_{2\alpha}$ on LH-stimulated progesterone secretion has been reported in both bovine (Alila *et al.*, 1988a), and ovine (Schwall, Sawyer & Niswender, 1986a), large luteal cells. Ovine large luteal cells fail to show any progesterone secretory response to cAMP (Hoyer & Kong, 1989) or dibutyryl cAMP (Fitz *et al.*, 1982) or to activators of adenylate cyclase (Hoyer *et al.*, 1984). In contrast bovine large luteal cells respond to LH, forskolin and 8-bromo-cAMP with an increase in progesterone production (Alila *et al.*, 1988a; Alila *et al.*, 1989; Hansel *et al.*, 1991). Activation of the protein kinase C second messenger system with the phorbol ester PMA is inhibitory to basal and LH-stimulated progesterone production in large and small luteal cells respectively (Hoyer & Marion, 1989;

Wiltbank, Knickerbocker & Niswender, 1989a). In contrast, phorbol esters elicit a steroidogenic response in small bovine luteal cells (Benhaim, Herrou, Mitre & Leymarie, 1987; Hansel *et al.*, 1987; Alila *et al.*, 1988a). LH caused no change in intracellular concentrations of free calcium in small or large ovine luteal cells, even though the dose of LH stimulated progesterone production by small luteal cells (Wiltbank, Guthrie, Mattson, Kater & Niswender, 1989b). However, transmembrane signalling of second messengers by LH in both small and large bovine luteal cells involves increases in $[Ca]_i$ (Davis *et al.*, 1987a; Davis, Alila, West, Corradino & Hansel, 1988; Hansel *et al.*, 1991).

1.6.2. Peptide synthesis

Oxytocin is a peptide hormone normally associated with hypothalamic-neurohypophyseal events at parturition and during lactation (Auletta & Flint, 1988). Oxytocin has been identified in the CL of cows, ewes and goats (Wathes & Swann, 1982; Fields, Eldridge, Fuchs, Roberts & Fields, 1983; Wathes, Swann, Birkett, Porter & Pickering, 1983; Wathes, Swann & Pickering, 1984; Schams, Kruip & Koll, 1985a; Homeida, 1986; Sawyer, Moeller & Kozlowski, 1986; Schams, Koll, Ivell, Mittermeier & Kruip, 1987). Its presence has been demonstrated by techniques including RIA (Flint & Sheldrick, 1982a), amino acid sequencing and various forms of chromatography (Flint, Sheldrick, McCann & Jones, 1990), and by identification of an uterine-contracting peptide in extracts of ovine and bovine luteal tissue (Fields, Fields, Castro-Hernandez & Larkin, 1980; Wathes & Swann, 1982; Fields *et al.*, 1983). Many lines of evidence indicate that oxytocin is synthesized (Swann, O'Shaughnessy, Birkett, Wathes, Porter & Pickering, 1984; Wathes, 1984), stored (Flint & Sheldrick, 1982a; Wathes & Swann, 1982), and secreted (Flint & Sheldrick, 1982b; Schams *et al.*, 1985a), by both ovine and bovine CL. Additional evidence for the secretion of oxytocin by ovine and bovine CL is

as follows: plasma oxytocin concentrations during the luteal phase decreased during luteolysis (Flint & Sheldrick, 1983; Schallenberger *et al.*, 1984); during the mid-luteal phase of the bovine oestrous cycle plasma oxytocin and progesterone concentrations were higher in the plasma from the inferior vena cava relative to the jugular vein (Walters *et al.*, 1984); and in sheep, plasma concentrations of oxytocin and its associated neurophysin were elevated in the ovarian vein relative to the ovarian artery (Flint, Sheldrick, Watkins & Moore, 1984).

In the ewe the large luteal cell is responsible for oxytocin synthesis and secretion (Rodgers, O'Shea, Findlay, Flint & Sheldrick, 1983b), and tissue oxytocin content correlates with the number of large luteal cells (Schwall, Gamboni, Mayan & Niswender, 1986b). In the cow the pattern of binding of granulosa antibody observed by Alila & Hansel (1984) closely resembled the profile of oxytocin secretion described by Walters *et al.*, 1984; the disappearance of granulosa cells during pregnancy coincides with the period when luteal oxytocin concentration is low, suggesting that granulosa-derived luteal cells are the source of oxytocin in the bovine CL. Immunohistochemical studies have indicated that oxytocin and its associated neurophysin are restricted to large luteal cells in sheep (Rodgers *et al.*, 1983b; Watkins, 1983), and cows (Guldenaar, Wathes & Pickering, 1984; Kruip, Vullings, Schams, Jonis & Klarenbeek, 1985). Granule loss by large luteal cells during luteal tissue dissociation is accompanied by a substantial fall in the oxytocin content of the large luteal cell (Rodgers *et al.*, 1983b), and PGF₂ α -induced release of oxytocin *in vivo* in ewes is associated with exocytosis of large numbers of secretory granules (Fairclough, Staples & O'Shea, 1985). Oxytocin and neurophysin have subsequently been localized to the 0.3 μ m diameter membrane-bound secretory granules in ewes (Theodosis, Wooding, Sheldrick & Flint, 1986), and cows (Fields & Fields, 1986; Fields,

Barros, Watkins & Fields, 1992). This co-localization reflects *de novo* synthesis of these peptides (Auletta & Flint, 1988). Moreover, the secretory granules have been shown to contain the enzymes involved in the post-translational processing of the prohormone (Clamagirand, Camier, Fahy, Clavreul, Creminon & Cohen, 1987). In the bovine (Ivell & Richter, 1984), and ovine (Jones & Flint, 1986) CL, as in the hypothalamus, oxytocin and neurophysin are produced from the same messenger RNA (mRNA) and cleaved from the prohormone after translation. Oxytocin prohormone mRNA has been cloned from, sequenced, and measured in both bovine and ovine CL (Ivell & Richter, 1984; Jones & Flint, 1988).

The follicle is able to secrete small amounts of oxytocin (Schams, 1987). Immunoreactive oxytocin was demonstrated immunocytochemically in small and large follicles (Kruip *et al.*, 1985), although Jungclas & Luck (1986) could not measure oxytocin in follicular fluid. The granulosa cells are the main source of follicular oxytocin (Geenen, Legros, Hazee-Hagelstein, Louis-Kohn, Lecomte-Yerna, Demoulin & Franchimont, 1985). Luteinization is the main stimulus for oxytocin secretion (Guldenaar *et al.*, 1984; Kruip *et al.*, 1985; Jungclas & Luck, 1986; Schams, 1987). Expression of the oxytocin gene in the bovine CL is first detected early in the oestrous cycle (Ivell, Brackett, Fields & Richter, 1985), and oxytocin mRNA content suggests that most oxytocin is synthesized during the early luteal phase (Schams, 1987). Secretion of oxytocin from the CL occurs throughout the luteal phase of the oestrous cycle (Sheldrick & Flint, 1981; Flint & Sheldrick, 1983; Walters *et al.*, 1984). Both oxytocin and neurophysin are secreted simultaneously (Schams, Schallenberger & Legros, 1985b). Changes in plasma concentrations of oxytocin and progesterone were parallel (Flint & Sheldrick, 1983; Sheldrick & Flint, 1984; Walters *et al.*, 1984) with pulses of oxytocin and progesterone occurring concomitantly (Schallenberger *et al.*, 1984).

Corpora lutea also produce relaxin in the pregnant cow and sow (Sherwood, 1988). Luteal cells from pregnant cows stained positively for relaxin with an immunohistochemical method that employed an anti-porcine relaxin serum (Fields *et al.*, 1980). However, there is no evidence that bovine luteal cells contain relaxin storage granules, and levels of relaxin bioactivity in extracts of CL from pregnant cows were reported to be very low (Fields *et al.*, 1980).

1.6.3. The role of secretory granules

Willcox, Jenkin, Quirk & Thorburn (1979) demonstrated the presence of a progesterone-binding protein in the CL, and several investigators (Quirk, Willcox, Parry & Thorburn, 1979; Parry *et al.*, 1980) have suggested that some of the progesterone in the bovine CL is sequestered within electron-dense granules, representing a mechanism by which progesterone is actively secreted. However there is no direct evidence that the secretory granules contain progesterone, with or without its binding protein, and others argue that once progesterone is synthesized it leaves the cell by diffusion (Enders, 1973). Furthermore, although both small and large bovine luteal cells secrete progesterone, only large cells have secretory granules (O'Shea, 1987). It has subsequently been established that oxytocin and neurophysin are major products of luteal secretory granules during the oestrous cycle but not of secretory granules during the post-implantation period of pregnancy (>Day 45) (Fields *et al.*, 1992). The number of granules and concentration of luteal oxytocin peak mid-cycle and then decrease precipitously. In pregnant cows, luteal granules of unknown contents rise from nearly undetectable numbers on Day 45 to a peak on Days 180-210.

1.6.4. Cell-cell communication

In the ewe small luteal cells are highly responsive to LH stimulation (Fitz *et al.*, 1982; Rodgers *et al.*, 1983a), and are the first to decline in number during PGF₂ α -induced luteal regression (Braden, Gamboni & Niswender, 1988). However PGF₂ α receptors are 30-fold higher in large luteal cells than in small luteal cells (Fitz *et al.*, 1982), suggesting that the initial site of action of PGF₂ α is on the large luteal cells which in turn release a substance that acts on the small luteal cells. Cell-cell communication can be defined as one cell producing a substance that in some way affects the function of the other cell. This message can potentially be transmitted by way of intercellular junctions, or extracellular matrix, or it may be humoral (Rodgers, 1990).

Progesterone production by isolated populations of porcine small and large luteal cells from pregnant sows was found to be almost doubled when these populations were co-incubated (Lemon & Mauléon, 1982). Harrison *et al.* (1987) have also observed a small enhancement of progesterone production when ovine small and large luteal cells from mid-cycle CL were co-cultured, but this effect has not been observed by others using either ovine (Rodgers *et al.*, 1983a; Rodgers, O'Shea & Findlay, 1985) or bovine (Ursely & Leymarie, 1979) luteal cells. The potential for oxytocin to act as a cell-cell mediator stems from the fact that in ruminants it is produced by one cell type, the large luteal cell (Rodgers *et al.*, 1983b; Guldenaar *et al.*, 1984; Fields & Fields, 1986). The LH-stimulated component of progesterone production is mainly a function of the small luteal cells (Fitz *et al.*, 1982; Rodgers *et al.*, 1983a) and oxytocin has been shown to inhibit progesterone production stimulated by LH (Tan, Tweedale & Biggs, 1982) suggesting that oxytocin may constitute a means of communication between large and small

luteal cells. Subsequently Rodgers *et al.* (1985) reported no effect of oxytocin on steroidogenesis by separated populations of ovine small and large luteal cells. Oxytocin receptors have been detected in cultured bovine luteal cells (Okuda, Miyamoto, Sauerwein, Schweigert & Schams, 1992) and in ovine CL from Day 30 of gestation onwards but could not be detected in CL of cyclic ewes (Sernia, Gemmell & Thomas, 1989).

In addition to the uterus prostaglandins are produced by CL (Milvae & Hansel, 1983; Rodgers, Mitchell & Simpson, 1988). Prostaglandins can alter luteal function *in vivo* (Horton & Poyser, 1976; Milvae & Hansel, 1980a; Gimenez & Henricks, 1983), and *in vitro*, including progesterone production (Rodgers *et al.*, 1985; Alila *et al.*, 1988a; Alila, Corradino & Hansel, 1988b; Pate & Nephew, 1988) and second messenger systems (Davis *et al.*, 1988). Prostaglandin receptors have been demonstrated in bovine CL (Powell, Hammarstrom & Samuelsson, 1975; Rao, 1975; Mattioli, Galeati & Seren, 1983).

1.7. Regulation of luteal function

In most species, three organ systems regulate the function of the CL (Niswender & Nett, 1988). The anterior pituitary gland secretes LH which is the primary hormone responsible for regulating the secretion of progesterone in most species (Niswender, Sawyer, Chen & Endres, 1980). In several rodent species, hypophysial prolactin is also an important regulator of luteal function but a luteotrophic role for prolactin in sheep and cattle has not been confirmed. In most non-primate species the uterus has a luteolytic effect during the late-luteal phase of the oestrous cycle. Finally the conceptus has either direct or indirect luteotrophic effects.

1.7.1. Hormones

1.7.1.1. GnRH

GnRH, by virtue of its ability to cause release of LH, can serve as a luteotrophic hormone. A long-acting GnRH analogue given frequently during the luteal phase of the bovine cycle produced prolonged increases in plasma progesterone concentrations and resulted in extension of the oestrous cycle (Hansel & Convey, 1983; Milvae *et al.*, 1984). Ovarian GnRH receptors have not been demonstrated in the sheep, cow or pig (Brown & Reeves, 1983), but Milvae *et al.*, (1984) demonstrated a direct effect of GnRH on the ovary of the cow. The inhibitory action of GnRH on luteal cells of the rat is very similar to that of PGF₂ α , which serves as a natural luteolysin (Williams & Behrman, 1983).

1.7.1.2. LH

There has been considerable controversy regarding the requirement for LH and/or prolactin, secreted by the anterior pituitary gland, for the regulation of luteal function in ruminants. Denamur & Mauléon (1963) reported that formation and maintenance of the CL in ewes was not influenced by hypophysectomy. However Kaltenbach, Graber, Niswender & Nalbandov (1968) found that hypophysectomy on Day 1 after a normal or induced ovulation resulted in failure of the CL to form, while hypophysectomy on Day 5 resulted in regression of the partially formed CL. Evidence reviewed by Rothchild (1981) suggests that for the first few days following ovulation the CL of most species continue a normal course of development and secrete progesterone unaided by external signals, referred to as "autonomous progesterone secretion". However the CL in any species

does not survive long in the absence of pituitary hormones. The term "luteotrophins" or "luteotropins" has been given to those pituitary or placental hormones that promote the growth of the CL and stimulate the secretion of progesterone (Astwood, 1941; Rothchild, 1981). For a given species there is a primary luteotrophic hormone, i.e. the hormone that maintains the gland as a viable entity and promotes progesterone secretion. There may also be secondary luteotrophic hormones, i.e. hormone(s) that can stimulate progesterone synthesis but that do not serve a vital role (Keyes *et al.*, 1983).

Further controversy stemmed from experiments designed to determine which hypophysial hormone(s) were responsible for the maintenance of luteal function. Injections of prolactin resulted in maintenance of luteal weight in hypophysectomized, hysterectomized ewes (Denamur, Martinet & Short, 1973; Thibault, 1966), but this hormone was without effect when infused into hypophysectomized ewes with an intact uterus (Kaltenbach *et al.*, 1968). Injections of LH would not maintain luteal weight in hypophysectomized, hysterectomized ewes (Denamur *et al.*, 1973), but infusion of LH was followed by maintenance of the CL in hypophysectomized ewes with an intact uterus (Kaltenbach *et al.*, 1968).

In the ewe Karsch, Roche, Noveroske, Foster, Norton & Nalbandov (1971) demonstrated that constant infusions of LH prolonged the lifespan and function of the CL in intact, cycling animals, and Fuller & Hansel (1970) showed that daily injections of antiserum to LH caused luteal regression in cycling ewes. In addition LH enhanced secretion of progesterone *in vivo* (Niswender *et al.*, 1976), and from luteal tissue *in vitro* (Simmons, Caffrey, Phillips, Abel & Niswender, 1976). It is probable that conflicting results from previous studies were due to the use of crude or partially-purified hormone

preparations, and it is now widely accepted that LH is critical for formation and maintenance of the CL in sheep.

Simmons & Hansel (1964) suggested LH was luteotrophic in the cow. Injections of LH lengthened the oestrous cycle (Donaldson & Hansel, 1965b), prevented the luteolytic effects of oxytocin injected early in the cycle (Simmons & Hansel, 1964; Donaldson, Hansel & Van Vleck, 1965), and increased concentrations of progesterone in CL from hysterectomized cows (Brunner, Donaldson & Hansel, 1969). Daily injections of LH antiserum reduced both luteal weight and content of progesterone in intact and hysterectomized heifers (Snook, Brunner, Saatman & Hansel, 1969), and LH stimulated synthesis and secretion of progesterone *in vivo* (Schomberg, Coudert & Short, 1967), and from luteal tissue *in vitro* (Armstrong & Black, 1966; Williams & Marsh, 1978; Hixon & Hansel, 1979). Hansel, Concannon & Lukaszewska (1973) concluded that LH was the primary luteotrophin in cattle.

In two species, the rat and the rabbit, LH has acute steroidogenic effects on luteal tissue, yet the direct action of LH on luteal cells is not required for the CL to function normally after ovulation (Bill & Keyes, 1983; Keyes, Possley & Brabec, 1987). However Rothchild (1981) reports a critical requirement for LH during Days 8-11 of pregnancy or pseudopregnancy in the rat; progesterone secretion fails in the absence of this hormone. LH has an essential indirect role; to stimulate the synthesis of oestrogen (Keyes *et al.*, 1983).

1.7.1.2.1. LH receptors

It has been generally accepted that the actions of protein hormones, including LH, are mediated via a specific receptor residing in the plasma membrane (Niswender *et al.*, 1985). Data from cell fractionation (Bramley &

Ryan, 1978a,b), have demonstrated that receptors for LH are localized in the plasma membrane of target cells. The presence of specific receptors for LH in the ovary and testis was first demonstrated by the ability of these tissues to preferentially bind and concentrate radioactively labelled LH or hCG *in vivo* (Channing & Kammerman, 1974). The LH receptor is a glycoprotein (Gospodarowicz, 1973) and has now been cloned from a number of species (Loosfelt, Misrahi, Atger, Salesse, Thi, Jolivet, Guiochon-Mantel, Sar, Jallal, Garnier & Milgrom, 1989; McFarland, Sprengel, Phillips, Kohler, Roseblit, Nikolics, Segaloff & Seeburg, 1989) and has been shown to be a member of the G-protein-coupled receptor family. The receptor is a single polypeptide which contains a large hydrophilic domain that is situated extracellularly and presumed to be involved in binding of large complex glycoproteins (Segaloff, Sprengel, Nikolics & Ascoli, 1990) followed by a peptide chain containing seven hydrophobic regions of 22-24 amino acids that span the plasma membrane, with the carboxy-terminal region being intracellular (Segaloff *et al.*, 1990).

Because plasma LH concentrations remain low during the luteal phase and do not parallel progesterone concentrations, an increase in LH receptor affinity or receptor number would be expected. In sheep (Diekman, O'Callaghan, Nett & Niswender, 1978a) and cattle (Rao, Estergreen, Carman & Moss, 1979) an increase in LH receptor affinity was not observed during the luteal phase. Most studies in which numbers of receptors for LH have been measured have utilized the binding of radioactive LH or hCG. It has been demonstrated that LH and hCG compete for the same specific receptor, and most studies have used hCG for a number of technical reasons (hCG can be radioiodinated to a higher specific activity and still retain its ability to bind to receptor; ^{125}I -labelled hCG is more stable than ^{125}I -labelled LH upon storage; and less non-specific binding occurs with hCG: Diekman *et al.*,

1978a). However it has become clear that the steroidogenic response, the time required for internalization of the hormone-receptor complex, and the lateral mobility in the membrane of the occupied LH receptor are different for LH and hCG (Niswender *et al.*, 1985). HCG has a prolonged half-life in blood compared to LH, (approximately 48 hours in the sheep compared to 25-30 minutes for LH). Furthermore, radioactive labelling methods fail to detect receptors occupied by endogenous hormone. However it is the number of receptors occupied by endogenous hormone which are responsible for the biological response at the time the sample was collected. Therefore, methods for quantification of both occupied and unoccupied receptors for LH were developed (Dickman *et al.*, 1978a). In ewes the total number of receptors for LH increased 40-fold between Days 2 and 14 of the cycle. There was a 6-fold increase in both the number of receptors occupied by endogenous hormone and the weight of the CL, and a 10-fold increase in serum progesterone concentrations during this period. However less than 0.5% of the total number of receptors was occupied by endogenous hormone. By Day 16 (late-luteal phase) both the total number of receptors and the number occupied by LH had decreased by 75% (Dickman *et al.*, 1978a). During the bovine oestrous cycle unoccupied luteal LH receptors increased during luteal development (Rao *et al.*, 1979; Spicer, Ireland & Roche, 1981; Garverick, Smith, Elmore, Morehouse, Agudo & Zahler, 1985), and decreased after luteal regression (Spicer *et al.*, 1981; Garverick *et al.*, 1985). During early pregnancy in the ewe the numbers of total and occupied receptors were very similar to those observed during the mid-luteal phase of the cycle (Dickman *et al.*, 1978a). An increase in the number of receptors, or number of receptors occupied by endogenous LH, was not associated with maintenance of the CL of pregnancy. However the decrease in number of receptors noted on Day 16 in cycling ewes did not occur. Following PGF₂ α administration to induce luteolysis, a 63% decrease in serum progesterone concentrations preceded a significant

decrease in LH receptors by 15 hours. It was concluded that a decrease in the number of receptors for LH was not involved in the initial luteolytic actions of PGF_{2α} (Diekman, O'Callaghan, Nett & Niswender, 1978b).

Throughout the oestrous cycle, the number of occupied and unoccupied receptors for LH were both highly correlated with the weight of the CL, serum concentrations of progesterone, and luteal concentrations of progesterone in sheep (Diekman *et al.*, 1978a) and cattle (Rao *et al.*, 1979; Spicer *et al.*, 1981; Garverick *et al.*, 1985). Maximum binding of hCG to bovine luteal tissue also occurred during the mid-luteal phase of the oestrous cycle (Spicer *et al.*, 1981). Thus in the cow and sheep secretion of progesterone is maximal when the number of luteal receptors for LH is highest, but when the circulating concentrations of LH are lowest during the cycle. The biological significance of the correlation between number of LH receptors and serum concentrations of progesterone is unclear. There is little correlation between episodic peaks of LH in serum and systemic concentrations of progesterone during the mid-luteal phase of the oestrous cycle in ewes (Baird, Swanston & Scaramuzzi, 1976a). When serum concentrations of LH were increased approximately 1000-fold during the mid-luteal phase of the oestrous cycle, the increase in serum concentrations of progesterone was less than 2-fold and lasted less than 6 hours (Suter, Fletcher, Sluss, Reichert & Niswender, 1980).

Less than 1% of receptors for LH in the ovary are occupied under conditions of maximal steroid secretion (Diekman *et al.*, 1978a). Circulating concentrations of LH are so low (0.5-1.5 ng/ml) that a large number of receptors is required to ensure that sufficient numbers are occupied by endogenous hormone to stimulate steroidogenesis (Niswender & Nett, 1988). A variety of factors may influence the concentration of LH receptors in the ovary (Richards, 1979), including oestradiol, FSH and LH. Exposure of luteal

tissue to high concentrations of LH or hCG results in an initial rapid increase in total number (occupied plus unoccupied) of LH receptors (up-regulation) followed by a dramatic loss (up to 90%) of LH receptors known as down-regulation (Suter *et al.*, 1980; Niswender *et al.*, 1985). A number of mechanisms have been suggested for recycling of hormone receptors and insertion into the plasma membrane which may explain up-regulation (Willingham & Pastan, 1984). Down-regulation is effected by internalization of the hormone-receptor complex via a receptor-mediated endocytosis (Niswender *et al.*, 1985) and the hormone is subsequently degraded by lysosomal enzymes. The receptor appears to be recycled to the plasma membrane (Suter & Niswender, 1983). Although the total number of receptors (occupied plus unoccupied) was decreased within 12-24 hours after the injection of LH, at no time during the study did serum concentrations of progesterone fall below preinjection levels, making the biological significance of down-regulation unclear (Niswender *et al.*, 1985). The number of receptors occupied at 10 minutes after injection of LH was highly correlated with the number of receptors lost by 24 hours suggesting that loss of LH receptors in ovine luteal cells is a function of occupancy by the hormone (Niswender *et al.*, 1985).

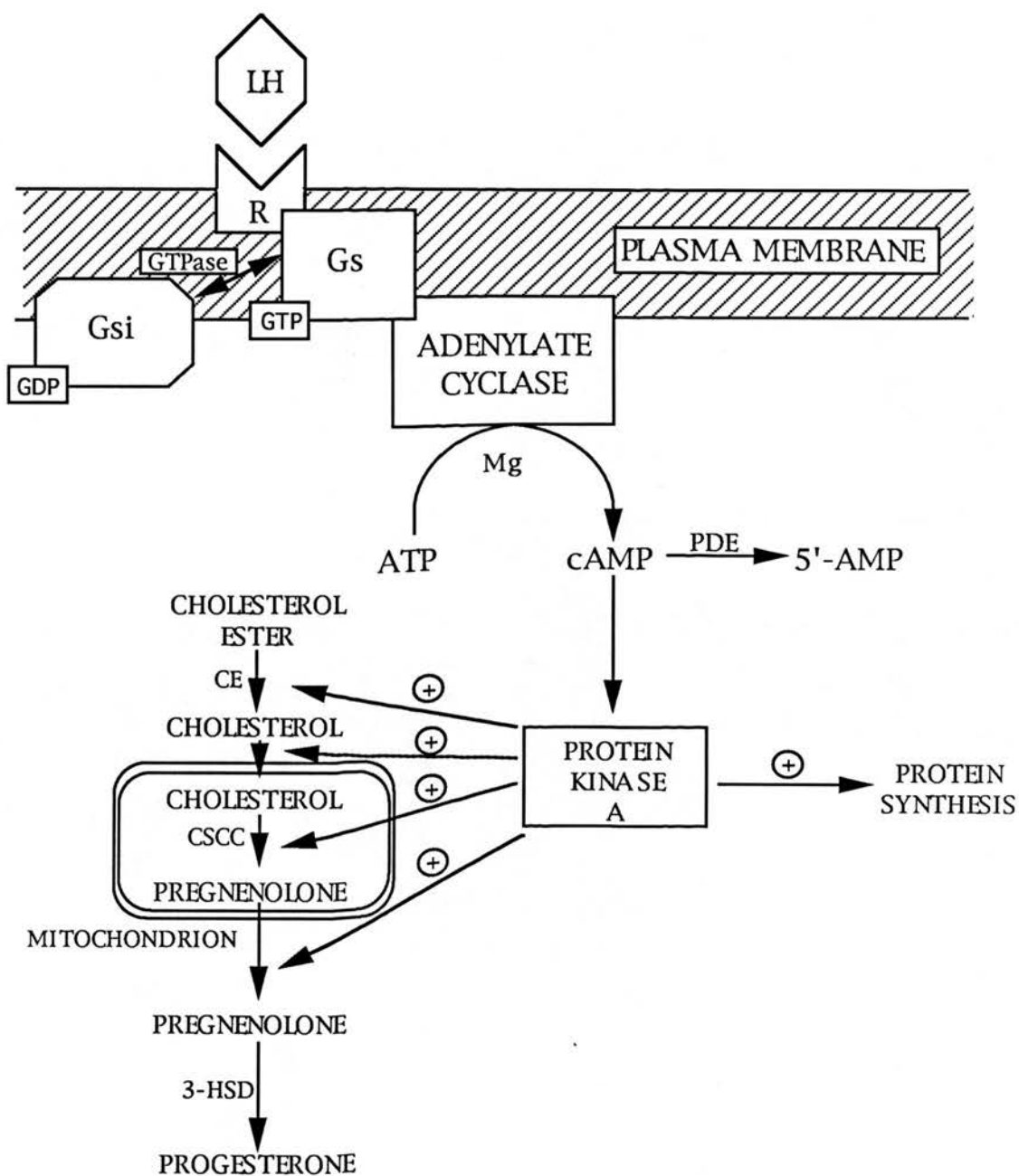
1.7.1.2.2. Mechanism of action of LH

LH exerts its steroidogenic effects on luteal tissue by increasing adenylate cyclase activity and intracellular cAMP concentrations (Marsh & Savard, 1966; Marsh, 1976; Ling, Williams & Marsh, 1980). In the bovine CL, LH and epinephrine are capable of activating adenylate cyclase (Condon & Black, 1976; Jordan, Caffrey & Niswender, 1978) which converts ATP to cAMP. cAMP then activates cAMP-dependent protein kinase, resulting in phosphorylation of an array of proteins, and leading to stimulation of steroidogenesis (Smith, 1986). The molecular events that occur as part of the

hormonal regulation of the biologic responses of target cells, have been described in detail (Niswender & Nett, 1988), and are summarized briefly (see Figure 1.1.). Following hormone binding, the hormone-receptor complex interacts with the regulatory G-protein, displacing bound guanosine diphosphate (GDP) and increasing guanosine triphosphate (GTP) binding. The G-protein in its GTP-bound state can activate the adenylate cyclase, which converts ATP to cAMP. Following hydrolysis of bound GTP to GDP, the G-protein dissociates from the adenylate cyclase and the enzyme is inactivated (Smith, 1986). Phosphodiesterase hydrolyzes cAMP to adenosine-5'-monophosphate (Thompson & Strada, 1978). cAMP is an important mediator of progesterone synthesis and the intracellular concentration of this nucleotide is regulated by changes in adenylate cyclase and phosphodiesterase activities (Marsh, 1976). In cattle luteal adenylate cyclase activity was correlated with progesterone secretion and increased during luteal development and decreased during luteolysis (Garverick *et al.*, 1985). Decreased phosphodiesterase activity was not involved in LH stimulation of progesterone secretion (Thompson & Strada, 1978); however, as CL aged, there was a concomitant increase in phosphodiesterase activity (Garverick *et al.*, 1985). In bovine CL, LH stimulated cAMP-dependent protein kinase activity. Intracellular cAMP activates protein kinase A and stimulates phosphorylation of a number of proteins, ultimately stimulating steroidogenesis. Darbon, Ursely & Leymarie (1976) and Ling & Marsh (1977) reported a positive correlation between stimulation of protein kinase activity and progesterone synthesis.

Increased protein kinase activity can influence the function of the luteal cell via several mechanisms (reviewed by Niswender & Nett, 1988). Protein kinases may enhance protein synthesis by phosphorylation of ribosomes; modulate enzyme activity via phosphorylation (including

Figure 1.1. Schematic representation of the events involved in LH-stimulated steroidogenesis. LH binds to its receptor (R) in the plasma membrane, which results in dissociation of GDP from the inactive G_i and binding of GTP, which activates the regulatory G-protein G_s. This results in activation of the catalytic subunit of adenylate cyclase which converts ATP to cAMP in the presence of Mg²⁺. cAMP activates protein kinase A. The active protein kinase A stimulates protein synthesis; activates cholesterol esterase (CE); activates cholesterol side-chain cleavage complex (CSCC); stimulates transport of cholesterol into the mitochondrion; and stimulates transport of pregnenolone out of the mitochondrion. Pregnenolone is converted to progesterone by 3 β -hydroxysteroid dehydrogenase/ Δ^5, Δ^4 -isomerase (3-HSD). GTPase inactivates G_s and phosphodiesterase (PDE) converts cAMP to 5'-AMP.



cholesterol esterase and cytochrome P-450 of cholesterol side-chain cleavage complex); enhance substrate availability via transport of cholesterol into mitochondria and/or by synthesis of cholesterol-binding protein; relieve end-product inhibition via transport of pregnenolone out of mitochondria; and play a role in the internalization and transport of receptors to lysosomes. LH was shown to stimulate *in vitro* endogenous cytosolic protein phosphorylation which was closely correlated with the stimulation of steroidogenesis (Darbon, Ursely, Mangué & Leymarie, 1980).

Finally cAMP may be involved directly in the chronic response of luteal cells to LH. Gospodarowicz & Gospodarowicz (1975) indicated that bovine luteal cells grown in culture tend to dedifferentiate with time but this was prevented by addition of LH or cAMP. LH-stimulated cAMP may be important for the differentiation of small to large luteal cells in ewes (Niswender *et al.*, 1985) and cows (Donaldson & Hansel, 1965a).

However there is evidence that not all of the luteotrophic effects of LH are mediated by cAMP. The activity of protein kinase and progesterone synthesis are enhanced in steroidogenic tissue at concentrations of LH that do not result in measurable increases in cAMP (Ling & Marsh, 1977), and progesterone responses to increasing concentrations of LH continued to rise after cAMP production had plateaued in bovine luteal slices (Hansel *et al.*, 1991).

Nishizuka (1984) described an alternative second messenger system, independent of cAMP, by which hormones can pass information from the surface to the interior of target cells by two routes; protein kinase C activation and intracellular calcium mobilization. Both routes are usually activated by the binding of the hormone to its receptor and subsequent phospholipase C hydrolysis of membrane-bound phosphatidylinositol 4,5-

bisphosphate (PIP_2), yielding diacylglycerol and IP_3 . The diacylglycerol activates a calcium-dependent protein kinase, protein kinase C, in the presence of membrane phospholipid, whilst the IP_3 mobilizes intracellular calcium. Both actions are usually necessary for a physiologic response. The rapid disappearance of diacylglycerol is due, in part, to its rapid hydrolysis to free fatty acids (including arachidonic acid which is the precursor of all the prostanoids; Hansel *et al.*, 1987) and to its re-phosphorylation to phosphatidic acid. Prostacyclin is a potent luteotrophin and its production in luteal cells is highly correlated with progesterone biosynthesis (Hansel & Dowd, 1986). Protein kinase C exerts its luteotrophic effect, in part, by stimulating the production of PGI_2 (Hansel *et al.*, 1987).

In bovine luteal tissue LH stimulated changes in phospholipid metabolism (primarily phosphatidylinositol) which were dose- and time-related to changes in progesterone secretion (Davis, Farese & Marsh, 1981). Davis & Clark (1983) identified a phospholipid-sensitive, calcium-dependent protein kinase in the CL of cattle. Subsequently Hansel & Dowd (1986) and Brunswig, Mukhopadhyay, Budnik, Bohnet & Leidenberger (1986) reported that the calcium-polyphosphoinositol-protein kinase C second messenger system was involved in the control of progesterone synthesis in the bovine CL. LH increased hydrolysis of membrane phosphatidylinositol via activation of phospholipase C (Davis *et al.*, 1987a). Pharmacologically protein kinase C can be activated by tumour-promoting phorbol esters such as PMA (Wiltbank *et al.*, 1989a) and phorbol dibutyrate (PBt_2) (Hansel *et al.*, 1987) which substitute for membrane-generated diacylglycerol (Brunswig *et al.*, 1986; Dowd, Alila & Hansel, 1990). The primary phorbol ester receptor in bovine luteal cells was shown to be protein kinase C (Hansel *et al.*, 1987; Dowd *et al.*, 1990). PMA synergised with suboptimal doses of hCG, 8-bromo-cAMP, cholera toxin (which activates the membrane G-protein by preventing hydrolysis of

GTP), and forskolin (which directly stimulates the catalytic subunit of adenylate cyclase), to increase progesterone production. However, PMA did not alter the conversion of pregnenolone or 25-hydroxycholesterol to progesterone, indicating a site of action distal to the formation of cAMP but before the formation of pregnenolone (Brunswig *et al.*, 1986). PMA enhanced protein phosphorylation in a membrane fraction of bovine luteal cells (Budnik & Mukhopadhyay, 1990). Treatment with phorbol esters has been reported to stimulate progesterone production in bovine luteal cells (Brunswig *et al.*, 1986); more specifically the steroidogenic response is a function of the small luteal cells (Benhaim *et al.*, 1987; Hansel *et al.*, 1987; Alila *et al.*, 1988a). Activation of protein kinase C had no effect on progesterone biosynthesis in large luteal cells (Hansel *et al.*, 1991). Furthermore PGF₂ α increased progesterone synthesis in bovine luteal cells *in vitro* (Hixon & Hansel, 1979), and enhanced progesterone synthesis stimulated by submaximal concentrations of LH (Hixon & Hansel, 1979), by stimulating phospholipase C and increasing phosphoinositide-derived second messengers (West, Weakland & Davis, 1986; Davis, Weakland, Weiland, Farese & West, 1987b) with resultant activation of protein kinase C (Davis *et al.*, 1988). The response was attributable to the small luteal cells (Davis *et al.*, 1989; Hansel *et al.*, 1991), and was rapidly desensitized (Hansel *et al.*, 1991), although the stimulus to prostanoid production continued for at least 24 hours (Hansel *et al.*, 1991).

Finally Hirata & Axelrod (1980) proposed phospholipid methylation as a second, possibly cAMP-independent, mechanism for signal transmission through the plasma membrane. Stimulation of progesterone secretion by LH was increased or inhibited when a stimulator (S-adenosyl methionine) or inhibitor (S-adenosyl homocysteine or 3-deazoadenosine) of phospholipid methylation was added to dispersed bovine luteal cells respectively (Milvae,

Alila & Hansel, 1983). Stimulation of progesterone secretion by either direct activation of adenylate cyclase with cholera toxin or addition of dibutyryl cAMP to dispersed bovine luteal cells was not altered by stimulators or inhibitors of phospholipid methylation, suggesting that the mechanism by which phospholipid methylation increased progesterone secretion either preceded, or was independent of, cAMP synthesis (Milvae *et al.*, 1983).

1.7.1.3. FSH

FSH receptors have been reported in the bovine CL (Manns, Niswender & Braden, 1984), and FSH stimulated progesterone secretion by perfused bovine luteal ovaries (Romanoff, 1966). From other studies there is evidence that FSH may be important in stimulating luteal progesterone production *in vivo* in the cow (Schallenberger, Rampp & Walters, 1983; Walters *et al.*, 1984), and pulses of FSH separate from LH pulses were observed which were associated with progesterone release. However Malven & Hansel (1964) noted that 10 daily injections of extracts of bovine hypophysial tissue incubated with urea (which reportedly destroys LH but not FSH), neither inhibited nor stimulated the persistent CL of hysterectomized heifers, whereas unincubated extracts increased CL weights and progesterone content. Moreover, FSH failed to stimulate progesterone secretion *in vitro* by bovine luteal slices (Hansel *et al.*, 1973), and failed to stimulate progesterone secretion when infused into the transplanted ovary of the ewe (McCracken, Baird & Goding, 1971). Finally, more recent work using GnRH agonist-treated sheep indicates that pulsatile progesterone release from the CL may be totally independent of pulsatile gonadotrophin secretion (McNeilly & Fraser, 1987).

1.7.1.4. Prolactin

Prolactin is the primary component of the luteotrophic complex in the rat (Astwood, 1941; Rothchild, 1981). However, although there is evidence that prolactin is important in the maintenance of the CL in hypophysectomized ewes, confirmatory data in intact animals is not available (for reviews see: Hansel *et al.*, 1973; Niswender *et al.*, 1985; Niswender & Nett, 1988). Administration of exogenous prolactin to cows does not alter the length of the oestrous cycle (Smith, McShan & Casida, 1957), prevent oxytocin-induced luteolysis (Donaldson *et al.*, 1965), or stimulate secretion of progesterone from luteal slices (Hansel *et al.*, 1973). Injections of 2-Br- α -ergocryptine (CB-154), which reduces serum concentrations of prolactin by more than 95%, did not reduce serum progesterone or influence the length of the bovine oestrous cycle (Hoffman, Schams, Bopp, Ender, Gimenez & Karg, 1974).

1.7.1.5. Oestrogen

Oestrogen is the only hormone required for a normal luteal phase in the rabbit (Bill & Keyes, 1983). Oestrogen is required by the rat for full progesterone secretion (Rothchild, 1981), although the luteal requirement for oestrogen may be met by testosterone which can be aromatized to oestradiol (Elbaum & Keyes, 1976). Oestrogen receptors are present in the sheep CL (Glass, Fitz & Niswender, 1984), and a specific oestradiol-17 β binding protein has been identified in the cytosol fraction of the bovine CL (Kimball & Hansel, 1974). However, exogenous oestrogens are luteolytic in the ewe and cow though they appear to be luteotrophic in sows (Kidder, Casida & Grummer, 1955; Gardner, First & Casida, 1963).

1.7.1.6. Catecholamines

Catecholamines may have an ancillary luteotrophic role. Catecholamines directly stimulate progesterone synthesis in bovine luteal tissue (Condon & Black, 1976; Godkin, Black & Duby, 1977), and dissociated ovine luteal cells (Jordan *et al.*, 1978). β -adrenergic receptors, in equal proportions of β 1- and β 2- types, have been found in membrane preparations of CL from pigs (Perkins, Cronin & Veldhuis, 1986). Spicer (1986) has reviewed catecholaminergic regulation of ovarian function in mammals.

1.7.1.7. Prostaglandins

PGE₂ is a likely candidate as a luteotrophic factor secreted from the pregnant uterus (Inskeep & Murdoch, 1980) because: (1) PGE₂ is a potent vasodilator and could overcome the vasoconstrictive effects of PGF₂ α (Bergstrom, Carlson & Weeks, 1968); (2) PGE₂ stimulates production of progesterone by luteal tissue *in vitro* (Marsh, 1971); both small and large luteal cells respond steroidogenically to PGE₂ (Alila *et al.*, 1988b); (3) simultaneous infusion of PGE₂ will prevent the decreased secretion of progesterone after treatment with PGF₂ α (Henderson, Scaramuzzi & Baird, 1977); (4) intrauterine administration of PGE₂ will prolong the oestrous cycle in ewes (Pratt, Butcher & Inskeep, 1977) and prevent natural and oestradiol-induced luteolysis (Colcord, Hoyer & Weems, 1978), whilst infusion of PGE₂ into the uterine lumen prolongs luteal lifespan in the cow for the duration of the infusion (Chenault, 1983); (5) PGE₂ secretion is greater from the pregnant than the non-pregnant uterus on Days 15-17 post-oestrus (Silvia, Ottobre & Inskeep, 1984a); and (6) secretion of PGE₂ by endometrial tissue *in vitro* is greater in pregnant than in non-pregnant ewes (LaCroix & Kann, 1982). Since PGF₂ α appears to be transferred from the utero-ovarian vein to the ovarian artery in ewes (McCracken *et al.*, 1971) and cows (Hixon &

Hansel, 1974), it seems likely that PGE₂ could reach the ovary via the same mechanism (Niswender *et al.*, 1985). PGF_{2α} has been shown to be cytotoxic to large luteal cells *in vitro*, an effect that is reversed by co-incubation in the presence of PGE₂ (Silvia, Fitz, Mayan & Niswender, 1984b).

Work in the cow has focussed on PGI₂ rather than prostaglandins of the E series. This difference in emphasis may be more apparent than real as it has been shown that PGE₁ binds to PGI₂ receptors (Lands, 1979). Bovine luteal tissue contains a large amount of prostacyclin synthetase which converts the prostaglandin endoperoxide to PGI₂ (Sun, Chapman & McGuire, 1977). Evidence for a luteotrophic role of PGI₂ may be summarized as follows: (1) injection of PGI₂ directly into the CL at mid-cycle produced a prolonged increase in peripheral plasma progesterone concentrations (Milvae & Hansel, 1980a); (2) PGI₂ stimulated progesterone synthesis by dispersed luteal cells *in vitro* (Milvae & Hansel, 1980a) and both small and large luteal cells respond steroidogenically to PGI₂ (Alila *et al.*, 1988b); (3) intrauterine administration of indomethacin, an inhibitor of prostaglandin synthesis, on Days 4-6 of the oestrous cycle, inhibited CL development and caused a reduction in oestrous cycle length (Milvae & Hansel, 1985); and (4) PGI₂ synthesis by luteal cells was greatest during Days 5-10 of the oestrous cycle, the period of early CL development (Milvae & Hansel, 1983), and subsequently declined. Factors that interfere with CL development during the early part of the bovine cycle (oxytocin administration, uterine irritants, certain bacterial and viral infections) may act by inhibiting either endometrial or luteal production of PGI₂ (Hansel & Convey, 1983). These workers suggest that CL function is regulated by a balance of luteotrophic and luteolytic prostaglandins.



1.7.1.8. Oxytocin

Oxytocin of luteal origin is involved indirectly in the regulation of luteal function and in luteolysis (see below). Evidence for a direct action of oxytocin on the CL from *in vitro* studies is inconclusive. In bovine luteal cells oxytocin has been observed to produce a minor stimulatory action on progesterone production at low doses and a marked suppression of hCG-stimulated progesterone production at higher doses in pregnant (Tan *et al.*, 1982) and non-pregnant (Tan & Biggs, 1984) cows. Oxytocin added to bovine luteal cells on Day 10 had little effect on progesterone synthesis (Milvae & Hansel, 1983), but oxytocin increased luteal progesterone secretion *in vitro* when cell-cell contact was maintained (Miyamoto & Schams, 1991) and Schams (1987) observed an inhibition of LH-stimulated steroidogenesis in luteal cells from Day 4-5 CL. In sheep oxytocin has been reported to inhibit LH-stimulated progesterone production by small luteal cells (Niswender *et al.*, 1985), although others were unable to observe any effect on large luteal or small luteal cell steroidogenesis (Rodgers *et al.*, 1985). There is apparently no local action in the CL late in the oestrous cycle because oxytocin is no longer synthesized or secreted at this time, nor when luteal lifespan is prolonged past the time of normal regression. However loss of oxytocin is not accompanied by any change in the secretion of progesterone, nor in the susceptibility of the CL to the luteolytic action of the synthetic PGF₂ α analogue, cloprostenol (Sheldrick & Flint, 1983a).

Since in some organs oxytocin is a vasoconstrictor (Linzell, 1974), the peptide may exert an inhibitory effect on luteal blood flow during luteal regression following release into venous effluent from large luteal cells (Auletta & Flint, 1988). Whether there are oxytocin receptors on the small

luteal cells is controversial (Tan *et al.*, 1982; Niswender *et al.*, 1985; Fuchs, Behrens, Helmer, Liv, Barros & Fields, 1990). It is possible that oxytocin receptors are on endothelial cells (Fitz *et al.*, 1982). If this is the case, it is uncertain whether the receptors would be involved directly in the metabolic responses of luteal cells (Auletta & Flint, 1988). The more recent demonstration of oxytocin/vasopressin receptors in the sheep CL (Sernia *et al.*, 1989) may not be inconsistent with the lack of a direct local effect of oxytocin on steroidogenesis, since these binding sites are expressed on vascular smooth muscle cells and may play a role in controlling blood flow (Flint & Sheldrick, 1982b).

1.7.2. Follicles

As has previously been described preovulatory extrafollicular and intrafollicular events can affect subsequent luteal lifespan and function. Ovarian follicles, via secretion of oestradiol, stimulate the secretion of PGF₂ α from the uterus. Destruction of follicles by X-irradiation delays CL regression in cows (Villa-Godoy, Ireland, Wortman, Ames & Fogwell, 1981; Fogwell, Cowley, Wortman, Ames & Ireland, 1985; Villa-Godoy, Ireland, Wortman, Ames, Hughes & Fogwell, 1985) and sheep (Karsch, Noveroske, Roche, Norton & Nalbandov, 1970), and prevents full CL regression in response to an injection of PGF₂ α (Hughes, Villa-Godoy, Kesner & Fogwell, 1987). The concentration of oestrogen in the utero-ovarian vein of treated cows was reduced following destruction of follicles (Fogwell *et al.*, 1985). Concentrations of LH were increased (Villa-Godoy *et al.*, 1985) or not different (Fogwell *et al.*, 1985) in treated animals compared to controls. Hughes *et al.* (1987) detected an increase in frequency and amplitude of LH pulses by Day 13 in irradiated heifers. An increase of similar magnitude in amplitude but not frequency of pulses of LH occurred in controls. A single injection of a GnRH agonist, which induces release of LH (Macmillan, Day,

Taufa, Gibb & Pearce, 1985a) increased the number of cloudy (atretic) follicles; however, daily progesterone concentrations in peripheral plasma from Day 12 until oestrus were not different from untreated animals (Thatcher, Macmillan, Hansen & Drost, 1989; Macmillan & Thatcher, 1991). Repeated injections of the GnRH agonist initiated on Day 12 and continued at 3-day intervals until Day 48 post-oestrus extended the interoestrus interval; plasma concentrations of progesterone were sustained throughout the treatment period (Thatcher *et al.*, 1989). During maternal recognition of pregnancy, ovarian follicular populations are altered (Guilbault, Dufour, Thatcher, Drost & Haibel, 1986) and follicular waves on the ovary bearing the CL but not on the contralateral ovary are suppressed (Pierson & Ginther, 1987c; Ginther, Kastelic & Knopf, 1989c).

1.7.3. Conceptus

Maternal recognition of pregnancy in ruminants involves physiological mechanisms that result in protection of the CL from luteolysis by modification or inhibition of uterine production of luteolytic pulses of $\text{PGF}_{2\alpha}$ (Bazer, Thatcher, Hansen, Mirando, Ott & Plante, 1991). Embryos can be successfully transferred as late as Day 12 post-oestrus in ewes (Moor & Rowson, 1966a) and Day 16 post-oestrus in cows (Betteridge, Egglesome, Randall & Mitchell, 1980) and the CL will be maintained. The lifespan of the CL is extended despite removal of ovine embryos on or after Day 13 (Moor & Rowson, 1966b) and bovine embryos on or after Day 17 (Northey & French, 1980). Thus the critical period for the maternal recognition of pregnancy in the cow is Day 16-17, and in the ewe is Day 12-13. When infused into the uterine lumen, homogenates of Day 14 to 16 ovine conceptuses (Ellinwood, Nett & Niswender, 1979) and Day 17 or 18 bovine embryos (Northey & French, 1980) prolong luteal function in sheep and cattle respectively. Infusion of a mixture of large MW conceptus secretory products into the uterine lumen

extended the interoestrous interval (Knickerbocker, Thatcher, Bazer, Drost, Barron, Fincher & Roberts, 1986a) and mimicked the suppressive effect of the embryo on secretion of $\text{PGF}_{2\alpha}$ from the uterus in response to treatment with oestradiol- 17β (Knickerbocker, Thatcher, Bazer, Barron & Roberts, 1986b). In addition to proteins, bovine and ovine conceptuses secrete $\text{PGF}_{2\alpha}$ and PGE_2 (Shemesh, Milaguir, Ayalon & Hansel, 1979). *In vitro* culture of sheep conceptuses and analysis of radiolabelled proteins resulted in identification of ovine trophoblast protein-1 (oTP-1) (see Bazer, 1989) as the anti-luteolytic agent. In cattle the conceptus protein that exerts the anti-luteolytic effect is bovine trophoblast protein-1 (bTP-1) (Helmer, Hansen, Anthony, Thatcher, Bazer & Roberts, 1987) which cross reacts immunologically with oTP-1. The period of bTP-1 secretion is maximal around Days 16-19 of pregnancy (Bartol, Roberts, Bazer, Lewis, Godkin & Thatcher, 1985); however, mRNA for bTP-1 can be detected as early as Day 12 (Farin, Hansen, McDonnell, Murphy & Farin, 1989b). Infusion of bTP-1 into the uterine lumen of cyclic cows between Days 15.5 and 21 (Thatcher, Hansen, Gross, Helmer, Plante & Bazer, 1988; Helmer, Hansen, Thatcher, Johnson & Bazer, 1989a) increased the interoestrous interval and extended the functional lifespan of the CL. Bovine trophoblast protein-1 decreases $\text{PGF}_{2\alpha}$ secretion and increases PGE_2 secretion from endometrial explants while also inducing an inhibitor of $\text{PGF}_{2\alpha}$ synthesis (Thatcher *et al.*, 1988; Helmer, Gross, Newton, Hansen & Thatcher, 1989b). Bovine trophoblast protein-1 has a high amino acid sequence homology with interferons of the alpha-II class (Imakawa, Hansen, Malathy, Anthony, Polites, Marotti & Roberts, 1989). Intrauterine or intramuscular (i.m.) administration of recombinant bovine interferon α class I type I (rbIFN α) can extend the length of the oestrous cycle and lifespan of the CL (Plante, Hansen & Thatcher, 1988; Plante, Hansen, Martinod, Siegenthaler, Thatcher, Pollard & Leslie, 1989). Oxytocin-induced 13,14-dihydro-15-keto-prostaglandin $\text{F}_{2\alpha}$ (PGFM) release is reduced

by administration of rbIFN α i.m. or into the uterine lumen (Plante, Thatcher & Hansen, 1991). However, Thatcher *et al.* (1988) showed that rbIFN α does not reduce endometrial secretion of PGF $_{2\alpha}$ but increases the secretion of PGE $_2$ from endometrial explants.

1.7.4. Other regulators of luteal function

There are other potential regulators of luteal function. Lipoproteins, carriers of blood cholesterol, enhance progesterone production and suppress *de novo* cholesterol synthesis in bovine luteal cells (Pate & Condon, 1989). There was no difference in the ability of LDL or HDL cholesterol to stimulate progesterone secretion (Carroll, Grummer & Mao, 1992). Insulin has trophic effects on bovine luteal cells *in vitro* (O'Shaughnessy & Wathes, 1985a). Inhibitors of LH binding have been demonstrated in extracts of sheep and goat CL (Tsafiriri, 1988).

Available evidence suggests that the preovulatory gonadotrophin surge is the stimulus for luteinization either by the permissive action of LH, enabling cells to differentiate spontaneously by the neutralization of inhibitory factors, or by the direct action of LH on the follicle by activation of the LH receptor (Keyes *et al.*, 1983). When granulosa cells from Graafian follicles are placed in tissue culture, they undergo varying degrees of hypertrophy and produce progesterone without a requirement for gonadotrophin in the medium (Rothchild, 1981). However Channing *et al.*, (1980a) reported that pig granulosa cells from large follicles luteinized morphologically and produced significant quantities of progesterone in tissue culture only if they were removed from animals in which the LH surge had started. Follicular fluid altered the responses of cultured granulosa cells to gonadotrophins *in vitro*. Responsiveness to gonadotrophins was not entirely due to gonadotrophin receptors but modified by locally-produced

hormonal regulators. Luteinization stimulator and luteinization inhibitor activity have been observed in follicular fluid (Ledwitz-Rigby & Rigby, 1981); fluid from mid-cycle bovine follicles decreased granulosa cell secretion of progesterone and prostaglandin *in vitro*, but fluid from preovulatory follicles was devoid of such inhibitory activity (Shemesh, 1979). Addition of follicular fluid from porcine follicles prevents the morphological and endocrine changes of luteinization in granulosa cells explanted from large porcine follicles (Ledwitz-Rigby, Rigby, Gay, Stetson, Young & Channing, 1977). Murdoch & Inskeep (1981) aspirated follicular fluid and oocytes from sheep and rabbit follicles. The follicles failed to luteinize, but if ovine LH was injected into the aspirated follicle or into follicles with follicular fluid and oocyte present, then luteinization was observed. The ability of the nucleotide derivative dbcAMP to stimulate luteinization (Miller & Keyes, 1974) led to the conclusion that the action of LH is mediated by cAMP.

1.8. Subnormal luteal function

Subnormal CL have been divided into two groups (Garverick, Zollers & Smith, 1992); CL having a short lifespan (Odde, Wood, Kiracofe, McKee & Kittok, 1980; Coleman & Dailey, 1983; Copelin, Smith, Garverick & Youngquist, 1987), and those having normal lifespans but reduced progesterone secretion (Pratt, Bernardinelli, Stevens & Inskeep, 1982; Coleman & Dailey, 1983). Short luteal phases occur in cattle and sheep during puberty, following first spontaneous and gonadotrophin-induced ovulations post-partum and at the start of the breeding season in anoestrous ewes (Lauderdale, 1986). When CL from ^{or sheep} cows anticipated to have short luteal phases, were collected on Day 5 prior to luteal regression, luteal weight, concentration of luteal progesterone, concentrations of occupied and unoccupied LH receptors, and basal and agonist-stimulated adenylate cyclase activity were not different

from CL of cows expected to have normal luteal phases (Smith, Garverick, Youngquist & Zahler, 1986; Hunter, Southee & Lamming, 1988; Braden, King, Odde & Niswender, 1989a). In addition the number of luteal cells, ratio of large to small cells, and number of $\text{PGF}_{2\alpha}$ receptors were similar between short-lived and normal CL (Braden *et al.*, 1989a). In other studies total number (O'Shea, Rodgers & Wright, 1984; Rutter, Carruthers & Manns, 1985) and size (O'Shea *et al.*, 1984) of large and small cells, and progesterone secretion in response to LH (Kesler, Weston, Pimental, Troxel, Vincent & Hixon, 1981a) were decreased in subnormal CL. Short-lived CL in cattle are not more sensitive to the luteolytic effects of $\text{PGF}_{2\alpha}$ (Copelin, Smith, Garverick, Youngquist, McVey & Inskeep, 1988). Mechanisms associated with subnormal luteal function may occur prior to or following ovulation, and include inadequate follicular development, decrease of luteotrophic support and/or premature release of a luteolysin.

1.8.1. Follicular phase mechanisms

Preparation of luteal cells for progesterone synthesis begins during the follicular phase (Hansel & Fortune, 1978). Corpora lutea are a continuation of follicular maturation, but ovulation is not a prerequisite for luteinization and progesterone secretion in sheep (Murdoch & Dunn, 1983) and cattle (Kesler, Elmore, Brown & Garverick, 1981b), and does not guarantee normal luteal development and function. Ramirez-Godinez *et al.* (1982) reported reduced concentrations of FSH but not LH during the 4 days preceding a short luteal phase. However concentrations and secretory patterns of FSH and LH during the preovulatory period were not different in post-partum beef cows which exhibited short compared to normal luteal phases (Garverick, Parfet, Lee, Copelin, Youngquist & Smith, 1988), and administration of FSH (Lishman, Allison, Fogwell, Butcher & Inskeep, 1979) to post-partum cows prior to induction of ovulation failed to enhance luteal

function. Administration of charcoal-extracted bFF following $\text{PGF}_{2\alpha}$ -induced luteolysis in ewes temporarily reduced concentrations of FSH (McNeilly, 1984) and delayed the onset of oestrus but did not result in subnormal luteal function (Miller, Critser, Rowe & Ginther, 1979). In cattle, bFF before or after $\text{PGF}_{2\alpha}$ -induced luteolysis delayed the onset of oestrus, but did not alter subsequent luteal function and did not significantly suppress FSH (Johnson & Smith, 1985). Garcia-Winder, Lewis, Deaver, Smith, Lewis & Inskeep (1986) reported a higher pulse frequency of LH during the follicular phase in cows having a normal compared with a short luteal phase. Pulsatile injections of GnRH (2 μg every 2 hours) for 72 hours prior to a GnRH challenge in post-partum anoestrous cows resulted in normal length cycles (Mollett *et al.*, 1983). A GnRH challenge preceded by similarly timed saline injections resulted in short cycles (Mollett *et al.*, 1983). In post-partum cows the first ovulatory surge of LH has been reported to be smaller (Schams, Schallenberger, Menzer, Stangl, Zottmeier, Hoffman & Karg, 1978) or not different (Manns, Humphrey, Flood, Mapletoft, Rawlings & Cheng, 1983) than the subsequent surge of LH; the duration of the surge of LH induced by a single injection of GnRH was shorter than the spontaneous surge of LH (Troxel, Kesler, Noble & Carlin, 1980). Progestin treatment prior to the injection of GnRH prolonged LH release and enhanced luteal function (Troxel & Kesler, 1984a).

Premature stimulation of ovulation in ewes by intrafollicular injection of LH or FSH reduced concentrations of progesterone during the subsequent luteal phase (Murdoch *et al.*, 1983). In post-partum cows preovulatory follicles anticipated to form subnormal CL were steroidogenically subfunctional (Garcia-Winder, Lewis & Inskeep, 1985); oestradiol concentrations in plasma or follicular fluid of animals with short luteal phases were reduced (Sheffel, Pratt, Ferrell & Inskeep, 1982; Garcia-

Winder, Lewis, Townsend & Inskeep, 1987; Garverick *et al.*, 1988; Braden, King, Odde & Niswender, 1989b). In sheep expected to have short luteal phases numbers of follicular receptors for LH/hCG in granulosa and thecal cells of preovulatory follicles were lower (Hunter, Southey, McLeod & Haresign, 1986).

1.8.2. Luteal phase mechanisms

Although LH is required for normal luteal development in cattle and sheep (Niswender & Nett, 1988), mean concentration, frequency, amplitude and duration of pulses of LH were similar for cows having short or normal luteal phases (Ramirez-Godinez *et al.*, 1982; Garcia-Winder *et al.*, 1986; Garverick *et al.*, 1988) and injections of hCG during the luteal phase did not reduce the incidence of short luteal phases (Carruthers, Manns & Rutter, 1986).

Garverick *et al.* (1992) concluded that short-lived CL were due to a premature release of $\text{PGF}_{2\alpha}$ from the uterus. When post-partum cows were hysterectomized prior to the first ovulation post-partum, CL were maintained and secretion of progesterone was similar to that observed during a normal oestrous cycle (Copelin *et al.*, 1987). Furthermore, luteal lifespan in cows anticipated to have short luteal phases was prolonged following intrauterine infusion of indomethacin (Troxel & Kesler, 1984b) and following active immunization against $\text{PGF}_{2\alpha}$ (Copelin, Smith, Keisler & Garverick, 1989). Oxytocin induced a release of $\text{PGF}_{2\alpha}$ on Day 5 of a short but not a normal oestrous cycle in post-partum beef cows, and the magnitude of the oxytocin-induced $\text{PGF}_{2\alpha}$ release was similar to the oxytocin-induced release of $\text{PGF}_{2\alpha}$ on Day 16 of a normal cycle (Zollers, Garverick & Smith, 1989). Zollers, Garverick, Youngquist, Ottobre, Silcox, Copelin & Smith (1991) showed that the source of the increased $\text{PGF}_{2\alpha}$ secretion was the uterus. Concentrations of

PGFM were elevated in the early post-partum period in dairy cows (Thatcher, Wilcox, Collier, Eley & Head, 1980), and plasma concentrations of $\text{PGF}_{2\alpha}$ in the vena cava were higher on Days 4-9 following first ovulation in cows having short compared with normal luteal phases (Cooper, Carver, Villeneuve, Silvia & Inskip, 1991). Progesterone pretreatment in post-partum cows resulted in normal luteal phases with the peak release of PGFM occurring later than in cows exhibiting short luteal phases (Troxel & Kesler, 1984a). Concentrations of endometrial oxytocin receptors were high on Day 1 in anoestrous ewes expected to have both short and normal oestrous cycles. However on Days 3 and 5 endometrial oxytocin binding sites were greater in ewes having a short luteal phase (Hunter, 1991).

1.9 Luteolysis

In his erudite review Rothchild (1981) is intrigued by the ephemerality of the CL; it has a finite lifespan, and, at the end of the luteal phase of the ovarian cycle or pregnancy, it undergoes a period of degeneration or involution known as "luteal regression" or "luteolysis". Functional regression is first detectable as the period when the luteal cells cease, or have a reduced capacity, to synthesize and secrete progesterone. Striking changes in the fine structure of luteal cells and their intracellular organization are observed during structural regression. These two phases are not separate but temporally and probably causally related, and should be viewed as a continuum (Keyes *et al.*, 1983).

1.9.1. Role of the uterus

Uterine involvement in the luteolytic process was first recognized by Loeb in 1923, when he observed that removal of the uterus in guinea pigs lengthened the lifespan of the CL. Maintenance of the CL also occurs following hysterectomy in cattle and sheep (Wiltbank & Casida, 1956;

Anderson, 1966). Removal of the CL of the cow either at hysterectomy (Malven & Hansel, 1964) or 75 days after hysterectomy (Anderson & Bowerman, 1963) is followed by ovulation and formation of a new CL, which in turn is maintained for an extended period of time. However hysterectomy does not result in absolute maintenance of the CL; there is a progressive decrease in luteal tissue weight, progesterone concentration and 20- β -hydroxyprogesterone concentration in 36-, 45- and 60-day old CL of hysterectomized heifers (Brunner *et al.*, 1969). That the effect of the uterus on the ovary was due to a local rather than a systemic action was demonstrated when surgical removal of the uterine horn adjacent to the ovary bearing the CL in cattle (Ginther, Woody, Mahajan, Janakiraman & Casida, 1967; Ginther, 1974,1981) and sheep (Moor & Rowson, 1966c) prevented luteal regression; regression of the CL occurred normally when the uterine horn contralateral to the CL was removed. Distention or irritation of the bovine uterus during the first 8 days of the oestrous cycle inhibits CL development and results in a shortened cycle (Hansel & Wagner, 1960) only if the uterine horn ipsilateral to the functional CL is dilated (Ginther, Woody, Janakiraman & Casida, 1966). However, in other studies, simultaneous removal of the uterine horn ipsilateral to the CL and the contralateral ovary was followed by CL regression and recurrent oestrous cycles in 30-80% of animals (Ward, Longwell, Kreider & Godke, 1976; Hansel & Convey, 1983). Autotransplantation of either the uterus or the ovary to the neck, while leaving the other organ *in situ*, results in extended luteal function in ewes (Goding, Harrison, Heap & Linzell, 1967). However, when the uterus and ovaries were transplanted as a unit, normal cyclic ovarian behaviour continued in ewes (Harrison, Heap & Linzell, 1968). Further evidence for a luteolytic role of the uterus was provided by the ovarian transplant experiments of McCracken *et al.*, (1971).

1.9.2. Luteolytic action of PGF_{2α}

1.9.2.1. Evidence that PGF_{2α} is the uterine luteolysin

In sheep and cows luteolysis is attributed to the uterine synthesis and secretion of PGF_{2α} (for reviews see Horton & Poyser, 1976; Niswender *et al.*, 1985). Babcock (1966) first suggested that prostaglandins might be luteolytic, and in 1969 the luteolytic action of PGF_{2α} was demonstrated in pseudopregnant rats (Pharris & Wyngarden, 1969). This initial observation was rapidly extended to other species, and PGF_{2α} has been shown to exert a luteolytic effect in cattle (Lauderdale, 1972; Liehr, Marion & Olson, 1972; Louis, Hafs & Morrow, 1972; Rowson, Tervit & Brand, 1972; Hansel *et al.*, 1973; Hafs, Louis, Noden & Oxender, 1974; Lauderdale, 1974) and sheep (McCracken, Glew & Scaramuzzi, 1970; McCracken *et al.*, 1971; Barrett, de Blockey, Brown, Cumming, Goding, Mole & Obst, 1971). Considerable evidence has accumulated to suggest that PGF_{2α} is the factor of uterine origin responsible for luteolysis in the large domestic species. Elevated concentrations of PGF_{2α} in uterine venous drainage (McCracken, Carlson, Glew, Goding, Baird, Green & Samuelsson, 1972; Thorburn, Cox, Currie, Restall & Schneider, 1972a; Nancarrow, Buckmaster, Chamley, Cox, Cumming, Cummins, Drinan, Findlay, Goding, Restall, Schneider & Thorburn, 1973; Baird, Land, Scaramuzzi & Wheeler, 1976b), uterine tissues (Wilson, Cenedella, Butcher & Inskeep, 1972; Shemesh & Hansel, 1975a) and uterine flushings (Bartol, Thatcher, Bazer, Kimball, Chenault, Wilcox & Roberts, 1981) coincide closely with the decline in blood progesterone and the period of expected luteolysis. Administration of PGF_{2α} to ewes during the luteal phase, in a manner that closely resembles physiological delivery of this agent, causes luteolysis (McCracken, Schramm, Barcikowski & Wilson, 1981), and infusion of PGF_{2α} into the arterial supply of the autotransplanted ovary preparation caused a rapid decline in

progesterone secretion rate, followed by a rise in oestrogen secretion rate and a peak in LH secretion rate shortly after the onset of oestrus (McCracken *et al.*, 1972). Neutralization of endogenous PGF_{2α} by active (Scaramuzzi, Baird, Wheeler & Land, 1973; Scaramuzzi & Baird, 1976) or passive (Fairclough, Smith & Peterson, 1976; Fairclough, Smith & McGowan, 1981) immunization against PGF_{2α} resulted in prolonged luteal maintenance. Inhibition of uterine prostaglandin production with indomethacin blocks spontaneous luteal regression (Lewis & Warren, 1977). However bovine luteal tissues *per se* produce relatively large amounts of all the prostanoids both *in vitro* and *in vivo* (Shemesh & Hansel, 1975b; Rexroad & Guthrie, 1979; Milvae & Hansel, 1983; Hansel & Dowd, 1986); thus the CL is not entirely dependent on prostaglandins of uterine origin for regression. Administration of PGF_{2α} to dispersed bovine luteal cells *in vitro* resulted in a small increase in progesterone production rather than the expected decrease (Hansel *et al.*, 1973; Hixon & Hansel, 1979). Hysterectomized cows appear to be more, rather than less, sensitive to exogenous PGF_{2α} than uterine-intact cows (LaVoie, Poncelet, Han, Soliday, Lambert & Moody, 1975; Hansel & Fortune, 1978) suggesting that the uterus may produce a luteotrophic substance as well as a luteolysin. Unlike the ewe, in which 99% of injected PGF_{2α} is metabolized in a single passage through the lungs, substantial amounts of injected PGF_{2α} pass through the lungs of the cow without metabolic breakdown. Davis, Fleet, Hansford, Harrison & Walker (1984) found that 35.0% of injected tritium-labelled PGF_{2α} survived a first passage through the lungs and 15.7% survived three circulations. Thus, PGF_{2α} can have a systemic effect in the cow. Endometrial and uterine venous plasma concentrations of PGF_{2α} are much higher in pregnant than in non-pregnant ewes at Day 13 (Wilson *et al.*, 1972) and plasma progesterone concentrations fall earlier than PGF_{2α} concentrations rise in pregnant ewes and goats just prior to parturition (Thorburn, Nicol, Bassett, Shutt & Cox, 1972b). Ultrastructural changes in

luteal cells following $\text{PGF}_{2\alpha}$ infusions did not mimic those that occur during natural luteolysis (Corteel, 1975). In particular, the increase in rough endoplasmic reticulum characteristic of normal regression did not occur.

1.9.2.2. Transport of the luteolytic substance

Local utero-ovarian relationships have been reviewed by Baird (1978). The first evidence for the operation of a countercurrent mechanism for transfer of a luteolysin of uterine origin from the uterine vein to the ovarian vein and then directly to the ovary by way of the ovarian artery was provided by Barrett *et al.* (1971) who interposed a piece of omentum between the ovarian artery and vein of the ewe resulting in maintenance of the CL. Furthermore, sectioning the broad ligament ipsilateral to the CL resulted in prolongation of the lifespan of the CL (Hixon & Hansel, 1974). Concentrations of $\text{PGF}_{2\alpha}$ were higher in the ovarian artery than in the carotid artery during a period of 40-120 minutes after intrauterine infusion of 6 mg $\text{PGF}_{2\alpha}$ (Hixon & Hansel, 1974). Uterine $\text{PGF}_{2\alpha}$ can be transferred locally from uterine lymphatic vessels to the adjacent ovary and ovarian vein (Heap, Fleet & Hamon, 1985). A series of elegant experiments utilizing surgical anastomoses of ovarian veins or ovarian arteries in hemihysterectomized cattle and sheep provided support for the functionality of the ovarian venous and arterial components in the local utero-ovarian pathway (Ginther, Del Campo & Rawlings, 1973; Ginther, 1974; Mapletoft, Del Campo & Ginther, 1976; Ginther, 1981). In cattle, sheep and pigs the ovarian artery is extremely convoluted and lies in close apposition with the ovarian vein which drains both the uterine horn and the adjacent ovary (Ginther, 1974, 1981). Although there do not appear to be vascular connections between the uterine vein and ovarian artery, there are regions of extensive contact between these two vessels throughout the broad ligament and up to their respective junctions with the vena cava and aorta (Ginther & Del Campo, 1974). Collateral channels and

venules separate from the uterine vein wrap around the ovarian artery, increasing the area of contact between the two vessels. The vessels share a common tunica adventitia and have thinner walls in regions where they make contact (Ginther & Del Campo, 1974).

However, some studies do not support the existence of a physiologically important veno-arterial transfer mechanism. Concentrations of $\text{PGF}_{2\alpha}$ in endometrium and uterine vein blood rose between Day 15 and 21, but $\text{PGF}_{2\alpha}$ in ovarian arterial blood did not increase (Shemesh & Hansel, 1975a). Milvae & Hansel (1980b) collected simultaneous and frequent blood samples from the uterine vein and ovarian artery following oxytocin injections during Days 4-6 of the cycle; each oxytocin injection was followed by a large and prolonged elevation in uterine vein $\text{PGF}_{2\alpha}$ concentration and a marked decline in jugular progesterone. However, no corresponding elevations in ovarian arterial $\text{PGF}_{2\alpha}$ concentrations were observed. Following infusions of [^3H]- $\text{PGF}_{2\alpha}$ into the uterine vein, the elevated radioactivity associated with $\text{PGF}_{2\alpha}$ in the ovarian arterial blood was maintained for about one hour after the infusion stopped, indicating a time lag in the transfer process, which would not be expected in a countercurrent mechanism (McCracken *et al.*, 1972). In sheep, sectioning the ovarian artery distal to the region where countercurrent transfer is thought to occur did not prevent luteal regression or prolong the cycle (Lamond & Drost, 1973). Land, Baird & Scaramuzzi (1976) found higher concentrations of $\text{PGF}_{2\alpha}$ in the ovarian artery than in the aorta at all stages of the cycle, and Coudert, Phillips, Faiman, Chernecki & Palmer (1974) concluded that veno-arterial transfer did not occur in ewes. However more recently McCracken *et al.* (1981); Thatcher, Wolfenson, Curl, Rico, Knickerbocker, Bazer & Drost (1984) and Wolfenson, Thatcher, Drost, Caton, Foster & LeBlanc (1985) demonstrated the exchange of $\text{PGF}_{2\alpha}$ from the

uterine venous drainage to the ovarian arterial supply in cattle and sheep, in keeping with the concept of a local veno-arterial pathway between the uterus and the ovary.

1.9.2.3. Mechanism of action of PGF_{2α}

In domestic livestock the luteolytic effects of PGF_{2α} are thought to occur in part through one or more direct actions on the luteal cells, and indirectly via a reduction in luteal blood flow (Knickerbocker, Wiltbank & Niswender, 1988).

1.9.2.3.1. Effects of PGF_{2α} at the cellular level

Specific receptors for PGF_{2α} have been identified in luteal cell membranes from cattle (Powell *et al.*, 1975; Rao, 1975; Rao *et al.*, 1979) and sheep (Powell, Hammarstrom & Samuelsson, 1974; Fitz *et al.*, 1982). Large luteal cells contain 10- and 30-fold higher concentrations of receptors for PGE₂ and PGF_{2α} respectively than small luteal cells (Fitz *et al.*, 1982; Niswender *et al.*, 1985). The action of PGF_{2α} may involve a reduction in the number of receptors for LH (Behrman, Grinwich, Hichens & MacDonald, 1978). However other studies in cattle and sheep have not detected a decrease in the number of luteal receptors for LH until well after a significant reduction in circulating concentrations of progesterone (Dieckman *et al.*, 1978b; Fitz, Fleeger, Smith & Harms, 1980; Rao, Ireland & Roche, 1984). X-ray diffraction studies on luteal tissue collected from cattle treated with PGF_{2α} and during spontaneous luteolysis exhibited reduced membrane fluidity. A portion of the lipid bilayer of the plasma membrane is in the gel phase at body temperature. In contrast, all microsomal membrane lipid from normal functional mid-cycle CL is in the liquid crystalline phase (Carlson, Buhr, Wentworth & Hansel, 1982; Goodsaid-Zalduondo, Rintoul, Carlson & Hansel, 1982). Thus, the ability of membrane proteins to associate and elicit

biological responses may be compromised during luteolysis by reducing their mobility within the cell membrane.

Addition of $\text{PGF}_{2\alpha}$ to dispersed bovine luteal cells increased progesterone synthesis (Hansel *et al.*, 1973; Hixon & Hansel, 1979; Weston & Hixon, 1980); this stimulation has been shown to be limited to the small theca-derived cells (Alila *et al.*, 1988a). With highly purified preparations of large luteal cells, $\text{PGF}_{2\alpha}$ had no effect on basal progesterone production (Alila *et al.*, 1988b), but inhibited LH-stimulated progesterone production in bovine (Alila *et al.*, 1988b) and ovine (Schwall *et al.*, 1986a) cells. A similar inhibitory effect of $\text{PGF}_{2\alpha}$ was observed on forskolin- and 8-bromo-cAMP-stimulated progesterone production in bovine large luteal cells (Alila *et al.*, 1988a). In bovine small luteal cell preparations, $\text{PGF}_{2\alpha}$ stimulated basal progesterone and LH-stimulated progesterone secretion (Alila *et al.*, 1988b). The stimulatory effect of $\text{PGF}_{2\alpha}$ is not found in ovine small luteal cells (Fletcher & Niswender, 1982). Because $\text{PGF}_{2\alpha}$ has no effect on LH-stimulated progesterone synthesis by ovine small luteal cells, but significantly inhibits progesterone production by small cells contaminated by large cells, it was concluded that the inhibition of LH-stimulated progesterone by small cells is dependent on the presence of the large luteal cells (Rodgers *et al.*, 1985).

Although it has been suggested that $\text{PGF}_{2\alpha}$ has a direct (inhibitory) effect on ovine luteal 3β -hydroxy-steroid dehydrogenase (Hoppen & Findlay, 1976) most mechanisms proposed for the inhibitory action of $\text{PGF}_{2\alpha}$ on luteal cells involve interference with gonadotrophin effects. The activity of a number of enzymes responsible for luteal steroidogenesis was generally not altered until after functional luteolysis has begun and the plasma concentration of progesterone had fallen (Rao *et al.*, 1984). $\text{PGF}_{2\alpha}$ is a potent antagonist of the action of LH or hCG in rat luteal minces or dispersed cells (Thomas, Dorflinger & Behrman, 1978; Behrman, Luborsky-Moore, Pang,

Wright & Dorflinger, 1979); this rapid inhibitory effect operated via a mechanism not mediated through interference with gonadotrophin binding (Thomas *et al.*, 1978). Fletcher & Niswender (1982) proposed that the luteolytic effect of $\text{PGF}_{2\alpha}$ in sheep involves an uncoupling of the LH receptor from adenylate cyclase. In bovine luteal cells a reduction in LH-activated adenylate cyclase activity results because the interaction between the regulatory and catalytic subunits of adenylate cyclase is altered (Pate & Condon, 1984).

The stimulatory effect of $\text{PGF}_{2\alpha}$ on bovine small luteal cells appears to be mediated by the phosphatidylinositide/calcium/protein kinase C second messenger system, rather than by an increase in cAMP (Davis *et al.*, 1988). Exposure of luteal cells from cattle (West *et al.*, 1986), sheep (Jacobs, Homanics & Silvia, 1987) and rats (Leung, Minegishi, Ma, Zhou & Ho-Yuen, 1986) to $\text{PGF}_{2\alpha}$ triggers a rapid decline in PIP_2 and concurrent increase in IP_3 . $\text{PGF}_{2\alpha}$ -stimulated progesterone production in small luteal cells requires calcium ions (Hansel *et al.*, 1991), and transmembrane signalling of second messengers by $\text{PGF}_{2\alpha}$ involves increases in $[\text{Ca}]_i$ in both small (Alila *et al.*, 1990) and large (Alila *et al.*, 1989) bovine luteal cells. The increase occurs in two phases, one representing mobilization of calcium from intracellular storage sites, and a secondary sustained rise representing influx of extracellular calcium (Hansel *et al.*, 1991). Additionally intracellular concentrations of calcium in large ovine luteal cells increase dramatically in response to $\text{PGF}_{2\alpha}$ whereas small luteal cells (which lack receptors for this hormone) are unaffected (Knickerbocker *et al.*, 1988). When large bovine luteal cells are treated with both LH and $\text{PGF}_{2\alpha}$, an increase in calcium occurs which is several times greater than the total of that induced by each hormone separately (Hansel *et al.*, 1991). Sustained elevations of intracellular free calcium concentrations are cytotoxic in numerous cell

systems (Rasmussen & Barret, 1984) suggesting that the luteolytic effects of $\text{PGF}_{2\alpha}$ are mediated in part by the increased intracellular concentration of calcium (Silvia *et al.*, 1984b). $\text{PGF}_{2\alpha}$ has been shown to reduce the activity of ion pumps in the CL which may allow high cytosolic calcium to persist (Kim & Yeoun, 1983). In bovine large luteal cells, LH-stimulated adenylate cyclase activity is inhibited by the calcium ionophore A23187 (Hansel & Dowd, 1986). $\text{PGF}_{2\alpha}$ induces intracellular concentrations of calcium sufficiently high to cause transport and exocytosis of oxytocin-containing secretory granules (Chegini & Rao, 1987).

Finally, the number of eosinophils (which are classically involved in the promotion of cellular injury) increases in luteal tissue following treatment of ewes with $\text{PGF}_{2\alpha}$ (Murdoch, 1987). In addition it has been shown that luteal extracts from $\text{PGF}_{2\alpha}$ -treated ewes, or leukotriene B₄, are chemoattractants for eosinophils *in vitro* (Murdoch, 1987). Eosinophil-mediated tissue damage may help to explain the inconsistency of $\text{PGF}_{2\alpha}$ effects on luteal cells *in vitro* versus the consistent luteolytic effects *in vivo*.

1.9.2.3.2. The vasculature during luteal regression

A second possible mechanism for the luteolytic action of $\text{PGF}_{2\alpha}$ involves a decrease in blood flow to the CL-bearing ovary. This was first thought to occur as a result of the known vasoconstrictor properties of $\text{PGF}_{2\alpha}$ (Pharris & Wyngarden, 1969). There is a dramatic reduction in ovarian and/or luteal blood flow associated with $\text{PGF}_{2\alpha}$ -induced luteolysis (Nett *et al.*, 1976; Nett & Niswender, 1981). A similar decrease in blood flow occurs during normal luteolysis (Niswender *et al.*, 1976; Ford & Chenault, 1981). Blood flow to the luteal ovary is correlated with peripheral progesterone concentrations during the oestrous cycle (Niswender *et al.*, 1976). In sheep, luteal blood flow fell from 11.2 ml/min/g luteal tissue on Day 14 of the oestrous cycle to 1.2

ml/min/g on Day 16 (Bruce & Moor, 1976). Ovarian blood flow in the cow dropped from 3.2 to 1.0 ml/min between Days -4 and -3 (oestrus=Day 0: Ford & Chenault, 1981). Changes in blood flow to the luteal ovary can be attributed to changes in flow to the CL (Niswender *et al.*, 1976). In addition, arterio-venous shunts or anastomoses appear to open up during luteal regression within the CL of the sheep that help to divert blood away from the capillaries (Ellinwood *et al.*, 1978). Arterio-venous shunts also exist in the ovarian stroma of sheep and may be responsible for redirecting blood away from the CL at the time of luteolysis (Mattner, Brown & Hales, 1981).

However the effect of $\text{PGF}_{2\alpha}$ on ovarian blood flow as a cause of luteal regression remains controversial. Although high doses of $\text{PGF}_{2\alpha}$ administered via the ovarian artery reduce ovarian blood flow to the autotransplanted ovary of the sheep (McCracken *et al.*, 1971), lower doses caused luteal regression in the absence of any blood flow changes (McCracken *et al.*, 1971).

Luteal blood flow has been reported to decrease prior to the decrease in progesterone secretion (Wehrenberg, Dierschke, Rankin & Wolf, 1978), and in other studies progesterone secretion decreases prior to a change in blood flow (Hossain, Lee, Clarke & O'Shea, 1979). Ellinwood *et al.* (1978) demonstrated that when a luteolytic dose of $\text{PGF}_{2\alpha}$ was administered to ewes on Day 9 of the oestrous cycle, a significant fall in blood flow to the ovary containing the CL was observed to precede by some 2 hours the first significant decline in serum progesterone concentrations. Nett & Niswender (1981) observed a 90% reduction in blood flow in the CL within 2 hours of $\text{PGF}_{2\alpha}$, preceding the decline in serum progesterone by some 4 hours and the decline in luteal LH receptor by more than 8 hours. However in the studies of McCracken *et al.* (1970) $\text{PGF}_{2\alpha}$ -induced diminution in progesterone secretion preceded changes in blood flow.

1.9.2.3.3. Morphological changes during luteal regression

Morphological changes associated with luteolysis are similar during spontaneous and PGF₂ α -induced regression (Knickerbocker *et al.*, 1988). Changes in the bovine CL include a decrease in cytoplasmic granulation, a rounding of the cell outline and peripheral vacuolation of the large luteal cells (Hansel *et al.*, 1973). These changes are rapidly followed by condensation of the cytoplasm which stains darkly and takes on a stellate outline. The first nuclear change noted is lighter staining and a loss of prominent nucleoli. Later, as the cytoplasm condenses, the nucleus shrinks, stains darkly and becomes pycnotic (Hansel *et al.*, 1973). Connective tissue elements become more prominent as regression continues. Early in luteolysis lipid droplet accumulation in the cytoplasm increases (Nett *et al.*, 1976; Parry *et al.*, 1980), while the number of protein-containing secretory granules declines. Additional early changes in the regressing CL appear in the capillaries (Nett *et al.*, 1976; Azmi & O'Shea, 1984). There is a dramatic loss in the number of endothelial cells which protrude into the capillary lumen, undergo nuclear and cytoplasmic condensation and fragmentation and are subsequently engulfed by viable endothelial cells (Azmi & O'Shea, 1984). An increase in cellular debris in the lumen of capillaries was associated with a decrease in percentage volume of the entire vascular compartment (Nett *et al.*, 1976; Niswender *et al.*, 1976). Lysosomes and autophagosomes increase in number as luteolysis progresses (Nett *et al.*, 1976) and the number of steroidogenic cells declines (Braden & Niswender, 1985; Farin *et al.*, 1986). Morphologically, the regressing CL appears to undergo apoptosis, a highly regulated cell death process that eliminates distinct populations of cells by activating endonucleases within the cells. Apoptosis, as indicated by oligonucleosome formation, was found to occur during both spontaneous and PGF₂ α -induced luteal regression in cattle, but was not apparent until after

serum progesterone concentrations had begun to decrease (Juengel, Garverick, Johnson, Youngquist & Smith, 1993).

1.9.2.4. PGF_{2α} and the establishment of pregnancy

From measurements of PGF_{2α} and PGFM, high amplitude surges in secretion which occur during and after luteolysis in non-pregnant animals are absent in pregnant ewes (Thorburn, Cox, Currie, Restall & Schneider, 1973; Barcikowski, Carlson, Wilson & McCracken, 1974; Zarco, Stabenfeldt, Kindahl, Bradford & Basu, 1984) and cows (Kindahl, Edqvist, Bane & Granstrom, 1976a). The concentration of PGF_{2α} in uterine venous plasma on Day 18 post-oestrus is significantly lower in pregnant cows compared to non-pregnant cows (Lukaszewska & Hansel, 1980). However the basal concentration of PGFM appears to increase during early pregnancy (Day 17 following breeding) in cattle (Williams, Lewis, Thatcher & Underwood, 1983; Thatcher *et al.*, 1984) and basal concentrations of PGF_{2α} in uterine venous plasma and basal concentrations of PGFM in peripheral plasma are greater in pregnant ewes (Days 12-18 after breeding: Zarco *et al.*, 1984). The concentrations of PGF_{2α} in uterine tissue and uterine luminal flushings are greater in pregnant than in non-pregnant ewes (Findlay, Colvin, Swaney & Doughton, 1983). *In vitro* secretion of PGF_{2α} from perfused endometrial tissue of pregnant cows at Day 17 after oestrus is markedly reduced and the tissue is unresponsive to treatment with oxytocin (Gross, Thatcher, Hansen & Lacroix, 1988a). When pregnant cows are injected with either oxytocin (Lafrance & Goff, 1985) or oestrogen (Thatcher *et al.*, 1984) PGF_{2α} release from the uterus is attenuated compared to the response of cyclic cows on the same day after oestrus. The reduced secretion of PGF_{2α} in endometrial tissue of early pregnancy is probably due to an intracellular endometrial prostaglandin synthetase inhibitor (Gross, Thatcher, Hansen, Johnson & Helmer, 1988b). Activity of the inhibitor was greater in the endometrial

tissue from Day 17 pregnant cows than in cyclic cows. Thus the gravid uterus retains the ability to synthesize $\text{PGF}_{2\alpha}$; however, the pattern of secretion appears to be altered. In addition, the sensitivity of the CL to the luteolytic action of $\text{PGF}_{2\alpha}$ appears to be altered in early pregnancy. Intrafollicular injections were less effective in inducing luteal regression in pregnant than in non-pregnant ewes (Inskeep, Smutny, Butcher & Pexton, 1975). The dose of $\text{PGF}_{2\alpha}$ required to induce continued luteolysis when given i.m., was less in cycling ewes than in pregnant ewes (Silvia & Niswender, 1986).

1.9.2.5. Role of arachidonic acid and its metabolites

In addition to prostaglandins of the F series (which are products of the cyclooxygenase pathway of arachidonic acid metabolism), evidence is accumulating for a role of arachidonic acid and the products of the lipoxygenase pathways of arachidonic acid metabolism in the luteolytic process (Hansel & Dowd, 1986; Hansel *et al.*, 1991). Early studies isolated and identified arachidonic acid as a luteolytic substance capable of inducing CL regression and decreasing plasma progesterone concentrations in pseudopregnant hysterectomized hamsters (Hansel, Shemesh, Hixon & Lukaszewska, 1975). Arachidonic acid injected directly into the bovine CL is rapidly converted into $\text{PGF}_{2\alpha}$ resulting in an immediate increase in ovarian venous $\text{PGF}_{2\alpha}$ and jugular oestrogen concentrations followed by a decrease in plasma progesterone (Shemesh & Hansel, 1975b). Bovine luteal and endometrial tissue contain relatively large amounts of arachidonic acid (Hansel *et al.*, 1973; Hansel *et al.*, 1975), but the concentration in endometrial tissue is only about one-sixth as high as in luteal tissue (Lukaszewska & Hansel, 1980). The source of these large amounts of arachidonic acid is not known, but part of the arachidonic acid found in the CL could be of endometrial origin (Hansel *et al.*, 1991). Bovine luteal tissue has a remarkable capacity to bind tritiated arachidonic acid which exceeds that in skeletal

muscle and ovarian stromal tissue by seven-fold (Shemesh & Hansel, 1975c). Additionally, high concentrations of 5-HETE, a product of the lipoxygenase pathway, were measured in luteal tissue collected during the mid- to late-luteal phase of the oestrous cycle, although synthesis of this product *in vitro* was not observed (Milvae, Alila & Hansel, 1986). Among the hydroxy fatty acid enzyme systems in the lipoxygenase pathway of arachidonic acid metabolism, of interest as potential luteolysins are the 5-lipoxygenase pathway which gives rise to the leukotrienes, and the 15-lipoxygenase pathway which gives rise to lipoxins A and B (Hansel & Dowd, 1986). In dispersed bovine luteal cells presence of the products of both the 5- and 15-lipoxygenase pathways caused a dose-related reduction in the synthesis of progesterone and PGI₂ (but not of PGF_{2α}), whereas 5-HETE alone, a compound of the 5-series, was less effective (Milvae *et al.*, 1986). Nordihydroguaiaretic acid (NDGA), a specific inhibitor of the lipoxygenase pathway, given twice daily to heifers between Days 14 to 18 of the cycle, delayed luteolysis and lengthened the oestrous cycle (Hansel & Dowd, 1986). When added to dispersed bovine luteal cells NDGA caused a slight increase in products of the cyclooxygenase pathway (Milvae *et al.*, 1986). In a subsequent study heifers were again given NDGA on Days 14-18. Luteal cells from CL collected after the last NDGA infusion contained (and produced) more prostaglandin F_{1α} (PGF_{1α}), a metabolite of prostaglandin F₁ (PGF₁), than did control cells (Milvae *et al.*, 1986), suggesting that the prolongation of luteal function was related to an increased ability of the CL of treated animals to synthesize PGI₂.

Arachidonic acid elicited a dose dependent increase in [Ca]_i in large and small luteal cells. As was the case for LH, there were two components of the arachidonic acid effects in small cells, but mobilization of calcium in large cells was mainly from the extracellular sources (Hansel *et al.*, 1991). Addition of inhibitors of the cyclooxygenase and lipoxygenase pathways of

arachidonic acid metabolism (indomethacin and NDGA respectively) were ineffective in suppressing the calcium-mobilizing actions of arachidonic acid. This suggests that mobilization of intracellular calcium was at least partly due to a direct action of arachidonic acid (Hansel *et al.*, 1991) and that arachidonic acid may have a direct luteolytic effect as a result of its ability to elevate intracellular calcium. Furthermore luteal regression and a decline in plasma progesterone concentrations could be induced by infusion of arachidonic acid directly into a branch of the ovarian artery of heifers, in which connections between ovarian pedicle and uterus were severed, resulting in prolonged maintenance of the CL (Hansel & Fortune, 1978).

1.9.3. Luteolytic action of oxytocin

Oxytocin may represent an intraluteal luteolytic signal. In the ewe LH receptors are present almost exclusively on the small luteal cells whereas $\text{PGF}_{2\alpha}$ receptors predominate on the large luteal cells (Fitz *et al.*, 1982; Niswender *et al.*, 1985). LH stimulates secretion of progesterone in small cells but has no effect on large luteal cells from cows (Ursely & Leymarie, 1979; Koos & Hansel, 1981) and ewes (Rodgers *et al.*, 1983a). Consistent with the hypothesis that oxytocin released from the large cells in response to $\text{PGF}_{2\alpha}$ exerts a lytic effect on the small cells, are the observations that $\text{PGF}_{2\alpha}$ stimulates release of oxytocin from the CL *in vivo* (Flint & Sheldrick, 1982b); that the pulsatile secretion of $\text{PGF}_{2\alpha}$ in utero-ovarian vein samples precedes the increase in oxytocin and neurophysin (Moore, Choy, Elliot & Watkins, 1986); and that the large cells are responsible for oxytocin synthesis and secretion (Rodgers *et al.*, 1983b; Theodosis *et al.*, 1986). However $\text{PGF}_{2\alpha}$ or cloprostenol failed to stimulate oxytocin secretion *in vitro* (Hirst, Rice, Jenkin & Thorburn, 1986, 1988).

Many studies provide evidence for an indirect involvement of oxytocin in the regulation of luteal function. In the cow injections of oxytocin in the early luteal phase inhibit CL development and shorten the oestrous cycle (Armstrong & Hansel, 1959; Hansel & Wagner, 1960; Black & Duby, 1965; Wilks & Hansel, 1971; Newcomb, Booth & Rowson, 1977; Milvae & Hansel, 1980b). This effect did not occur in completely hysterectomized animals or in animals in which the uterine horn ipsilateral to the CL was removed (Armstrong & Hansel, 1959; Ginther *et al.*, 1967; Brunner *et al.*, 1969; Newcomb *et al.*, 1977). Exogenous oxytocin does not appear to cause luteal regression in ewes although it may be followed by a slight depression in progesterone secretion (Hatjiminaoglou, Alifakiotis & Zervas, 1979). However continuous intravenous infusion of oxytocin (3 µg/hour) into ewes between Days 13-21 of the cycle maintained luteal function and lengthened the cycle by 7 days (Flint & Sheldrick, 1985). In heifers constant infusion of oxytocin from Day 10 (Lutz, Smith, Keisler & Garverick, 1991) caused prolongation of the oestrous cycle. In sheep both active (Sheldrick, Mitchell & Flint, 1980) and passive (Schams, Prokopp & Barth, 1983a) immunization against oxytocin delays luteolysis, although oxytocin antibodies had no effect on cyclicity in some ewes (Schams *et al.*, 1983a) and passive immunization of heifers did not prolong the oestrous cycle (Schams, 1987).

Administration of oxytocin in the early luteal phase has no effect on plasma LH concentrations (Wilks & Hansel, 1971) indicating that the oxytocin effect is not a result of reduced gonadotrophin secretion. Oxytocin treatment during the early luteal phase depresses the ability of the CL to produce the potent luteotrophin PGI₂, either by inhibition of enzymes necessary for prostacyclin synthesis, or excessive production of lipxygenase products, such as 5-HETE, which inhibit prostacyclin synthesis (Hansel & Dowd, 1986). Oxytocin treatment decreased the concentration of progesterone in jugular

venous blood by Day 8 in heifers (Milvae & Hansel, 1980b). Oxytocin increased uterine secretion of $\text{PGF}_{2\alpha}$ *in vivo* in cattle (Newcomb *et al.*, 1977; Milvae & Hansel, 1980b; Oyedipe, Gustafsson & Kindahl, 1984; Lafrance & Goff, 1985; Silvia & Taylor, 1989) and sheep (Mitchell, Flint & Turnbull, 1975; Fairclough, Moore, Peterson & Watkins, 1984) and from ovine endometrial tissue *in vitro* (Roberts, McCracken, Gavagan & Soloff, 1976), although the magnitude of the response varied according to the stage of the cycle. Uterine secretory responsiveness to oxytocin appears to develop late in the oestrous cycle at about the same time that pulsatile secretion of $\text{PGF}_{2\alpha}$ and luteolysis begin. This responsiveness is maintained into the early portion of the next oestrous cycle (Roberts *et al.*, 1976; Silvia & Taylor, 1989).

Concentrations of endometrial oxytocin receptor change during the oestrous cycle, reaching a maximum at oestrus and declining to almost undetectable levels during the mid-luteal phase before increasing again on Days 14-15 of the cycle in ewes (Roberts *et al.*, 1976; McCracken, 1980; Sheldrick & Flint, 1985) or Days 18-19 of the cycle in cows (Schams *et al.*, 1987). An increase in $\text{PGF}_{2\alpha}$ release in response to oxytocin was maximal on Day 3 in heifers (Newcomb *et al.*, 1977). Simultaneous treatment with a prostaglandin synthetase inhibitor in the goat (Cooke & Homeida, 1983) prevented the prostaglandin response to oxytocin, and systemic treatment with indomethacin in ewes inhibited pulsatile release of oxytocin (Watkins, Moore, Fairclough, Peterson & Tervit, 1984). Thus, it appears that the luteolytic action of oxytocin is mediated by $\text{PGF}_{2\alpha}$. Oxytocin released from the CL reaches the uterus where it stimulates release of $\text{PGF}_{2\alpha}$ which induces luteal regression (McCracken, Schramm & Okulicz, 1984), although exogenous oxytocin at mid-cycle or later does not cause premature luteal regression (Hansel & Wagner, 1960), and indomethacin infused into the uterine lumen was without significant effect on oxytocin-induced inhibition

of luteal function in the cow (Milvae & Hansel, 1985). Furthermore a transfer of $\text{PGF}_{2\alpha}$ from the uterine vein to the ovarian artery following an injection of oxytocin was not detected in cattle (Milvae & Hansel, 1980b). Hansel & Dowd (1986) suggest that large amounts of oxytocin administered during the critical period (Days 4-6) may inhibit CL development and progesterone secretion by interfering with the normal mechanisms for oxytocin synthesis and release; expression of the oxytocin gene is first detected early in the oestrous cycle at approximately the same time that administration of large amounts of oxytocin inhibit luteal function (Ivell *et al.*, 1985).

Conversely the administration of a luteolytic dose of $\text{PGF}_{2\alpha}$ stimulates luteal oxytocin secretion (Flint & Sheldrick, 1983; Schallenberger *et al.*, 1984). The $\text{PGF}_{2\alpha}$ analogue, cloprostenol, and the PGE_2 analogue, 15-methyl,15(S)- PGE_2 , will also stimulate luteal oxytocin secretion (Flint & Sheldrick, 1982b; Sheldrick & Flint, 1984), and $\text{PGF}_{2\alpha}$ administered into the uterine vein or draining lymph vessels is effective in stimulating oxytocin release (Heap, Fleet, Davis, Goode, Hamon, Walters & Flint, 1989).

Release of oxytocin and its associated neurophysin is either reduced (Sheldrick & Flint, 1981; Webb, Mitchell, Falconer & Robinson, 1981b; Moore, Watkins, Peterson, Tervit, Fairclough, Havik & Smith, 1982; Wathes *et al.*, 1983) or not different (Hooper, Watkins & Thorburn, 1986) in pregnant compared to cyclic ewes. During early pregnancy episodic secretion of oxytocin is reduced in amplitude (Fairclough *et al.*, 1984) but not in frequency; many episodes of oxytocin occur without $\text{PGF}_{2\alpha}$ (Hooper *et al.*, 1986). Luteal oxytocin concentrations are lower in pregnant than in non-pregnant ewes (Sheldrick & Flint, 1983b) and cows (Wathes *et al.*, 1983; Schams *et al.*, 1985a). The decline of ovarian oxytocin does not appear to be controlled by the presence of the conceptus because luteal concentrations were low in ovine CL maintained beyond the normal time of luteal regression

by hysterectomy (Sheldrick & Flint, 1983a). Sheldrick & Flint (1984) proposed that the decrease in luteal oxytocin concentration may be important for luteal maintenance. However, the loss of oxytocin is not accompanied by any change in the susceptibility of the CL to the luteolytic action of cloprostenol (Sheldrick & Flint, 1983a). Endometrial oxytocin receptor concentrations are very low or absent in pregnant ewes (McCracken *et al.*, 1981; Flint & Sheldrick, 1986).

1.9.4. Role of other hormones

LH appears to be the major luteotrophic principle in ruminants and there is little evidence that it can cause luteolysis as it does in the rabbit (Hansel *et al.*, 1973). Neither FSH nor prolactin have luteolytic effects (Malven & Hansel, 1964) although gonadotrophins, or combinations of gonadotrophins, that cause increased oestrogen secretion may have a luteolytic effect.

Evidence is accumulating for a role of oestrogens in luteal demise. Systemically administered oestradiol given during the mid-luteal phase of the oestrous cycle causes premature luteolysis in sheep (Hawk & Bolt, 1970; Gengenbach, Hixon & Hansel, 1977) and cows (Greenstein, Murray & Foley, 1958; Brunner *et al.*, 1969; Hansel *et al.*, 1973). Treatment of heifers with 200 µg oestradiol benzoate on Days 10-12 of the cycle, abolished the stimulatory effects of LH added *in vitro*, but had no significant effect on CL weights and progesterone concentration, and there was no change in plasma progesterone and LH concentrations, indicating that the luteolytic effect of oestradiol is not mediated by changes in plasma LH in ruminants (Hansel *et al.*, 1973), nor by inhibition of the steroidogenic response to LH (Hixon, Pimentel, Weston, Chafetz, Shanks & Hansel, 1983). Complete luteal regression was induced in intact heifers by a dose of oestradiol that produced only a

small non-significant decline in weight and total progesterone content in CL of hysterectomized animals (Wiltbank, 1966; Brunner *et al.*, 1969). Thus, a large part of the luteolytic action of oestradiol requires the presence of the uterus. Destruction of ovarian follicles, the prime source of oestrogen (for review see Hansel & Convey, 1983) by X-irradiation, prolongs luteal function in cows (Villa-Godoy *et al.*, 1981) and sheep (Karsch *et al.*, 1970).

The primary luteolytic action of oestradiol appears to be indirect and operates by causing the release of $\text{PGF}_{2\alpha}$ from the uterus (McCracken *et al.*, 1971; Hansel *et al.*, 1973; Ford, Weems, Pitts, Pexton, Butcher & Inskeep, 1975; Hixon & Flint, 1987). Acute injections of oestradiol to intact animals will induce secretion of $\text{PGF}_{2\alpha}$ within 6-8 hours (Barcikowski *et al.*, 1974; Knickerbocker, Thatcher, Foster, Wolfenson, Bartol & Caton, 1986c; Thatcher, Terqui, Thimonier & Mauléon, 1986) and episodic secretion of $\text{PGF}_{2\alpha}$ in 35 hours (Hixon & Flint, 1987). A similar response to oestradiol has been observed in ovariectomized animals receiving progesterone replacement for five days or more (Lafrance & Goff, 1988). The increase in $\text{PGF}_{2\alpha}$ secretion that follows cloprostenol-induced luteolysis in heifers is suppressed by treatment with an antagonist of oestradiol (Jacobs, Edgerton, Silvia & Schillo, 1988).

The luteolytic effects of oestradiol and $\text{PGF}_{2\alpha}$ are additive in sheep (Hixon, Gengenbach & Hansel, 1975) and cattle (Hixon *et al.*, 1983). This potentiating effect may be independent of the uterus (Gengenbach *et al.*, 1977); however, evidence for a direct action of oestradiol on bovine luteal cells *in vitro* was obtained only when very high concentrations (5-10 $\mu\text{g/ml}$) were used (Williams & Marsh, 1978). Although the bovine CL has been reported to possess cytosolic oestrogen binding proteins (Kimball & Hansel, 1974), the endogenous oestrogen content of bovine luteal tissue is extremely low as a consequence of very low or non-existent aromatase activity

(Henderson & Moon, 1979). Oestrogen may affect activity of enzymes involved in the synthesis of $\text{PGF}_{2\alpha}$ (Silvia, Lewis, McCracken, Thatcher & Wilson, 1991).

Administration of exogenous progesterone early in the cycle before progesterone rises, shortens the length of the oestrous cycle (Ottobre, Lewis, Thayne & Inskip, 1980) and reduces CL weights in the cow and ewe (Woody, First & Pope, 1967). There was a greater reduction in oestrous cycle length when progesterone injections were started on the day of oestrus in heifers than when started 2 days later (Woody & Ginther, 1968). Progesterone given at this time may act by inhibiting the relatively small rise in plasma LH that occurs on Days 3-4 of the cycle (Wilks & Hansel, 1971). However Woody & Ginther (1968) showed that the oestrous cycle-shortening effect of progesterone administered during the early part of the oestrous cycle requires the presence of the uterine horn ipsilateral to the CL. Administration of the progesterone receptor antagonist RU486 to ewes during the first half of the oestrous cycle delays the onset of pulsatile secretion of $\text{PGF}_{2\alpha}$ and luteal regression (Silvia *et al.*, 1991). Active immunization of ewes against progesterone results in erratic cyclicity and is frequently associated with prolonged maintenance of the CL (French & Spenetta, 1981).

1.9.5. Endocrine regulation of uterine secretion of $\text{PGF}_{2\alpha}$

In ruminants, interactions between ovarian oestradiol, progesterone and oxytocin with the uterine endometrium appear to regulate the timing and magnitude of $\text{PGF}_{2\alpha}$ production during the oestrous cycle (Knickerbocker *et al.*, 1988).

Prostaglandins have been detected in increasing concentrations in the blood at the time of luteolysis (Horton & Poyser, 1976). Measurement of

PGF_{2α}, or its major metabolite PGFM, in peripheral or utero-ovarian venous blood in sheep (Thorburn *et al.*, 1973; Baird *et al.*, 1976b) and cows (Kindahl, Granstrom, Edqvist & Eneroth, 1976b) has revealed pulses of secretion during luteolysis with a concomitant decrease in circulating progesterone. The pulses tend to increase in amplitude as the concentration of progesterone in the blood declines (Nancarrow *et al.*, 1973; Thorburn *et al.*, 1973). Typically there is a series of 5-8 discrete pulses (Nancarrow *et al.*, 1973; Peterson, Fairclough, Payne & Smith, 1975; Kindahl, Edqvist, Granstrom & Bane, 1976c) at 6-8 hour intervals. Maximum luteolytic response to administered PGF_{2α} is obtained when it is given at 6-hour intervals (Schramm, Bovaird, Glew, Schramm & McCracken, 1983). Pulses first appear just prior to the onset of luteal regression (Zarco, Stabenfeldt, Quirke, Kindahl & Bradford, 1988), although in some studies the initial decline in circulating concentrations of progesterone appears to precede any detectable pulses of PGF_{2α} in the blood (Stabenfeldt, Kindahl, Hughes, Neely, Liu & Pascoe, 1981). The major release of PGF_{2α} from the uterus follows rather than precedes the commencement of luteolysis (Thorburn *et al.*, 1973; Flint & Sheldrick, 1983). Several possibilities may explain the failure to detect consistently increased pulsatile release of PGF_{2α} prior to significantly reduced concentrations of progesterone. The site and frequency of blood sampling may have precluded the detection of episodic release of PGF_{2α} during the initial phase of luteolysis (Baird *et al.*, 1976b; McCracken *et al.*, 1981). On the other hand, PGF_{2α} concentrations in uterine vein plasma on any given day are rather variable perhaps because handling of the uterus incidental to collection causes PGF_{2α} release (Hansel *et al.*, 1973). The CL is refractory to PGF_{2α} early in the oestrous cycle (Rothchild, 1981) and becomes responsive to the luteolysin as the CL matures and acquires receptors for PGF_{2α} (Rao *et al.*, 1979; Fitz *et al.*, 1982). Thus, increased luteal sensitivity to PGF_{2α} during the later phases of the oestrous cycle may trigger luteolysis. However, exogenous PGF_{2α} is luteolytic as early

as Days 5-7 of the oestrous cycle in both cattle and sheep (Hafs *et al.*, 1974; Lauderdale, 1974). Moreover, the reduction in ovarian blood flow associated with luteolysis and augmented by the vasoconstrictive effects of $\text{PGF}_{2\alpha}$ (Pharris, Cornette & Gutknecht, 1970) would decrease perfusion of the CL and reduce transport of progesterone into the general circulation (Knickerbocker *et al.*, 1988).

Oxytocin concentrations increase in the blood during $\text{PGF}_{2\alpha}$ -induced luteolysis (Schallenberger *et al.*, 1984); simultaneous episodic secretion of oxytocin-neurophysin and $\text{PGF}_{2\alpha}$ occurs in sheep (Webb *et al.*, 1981b; Flint & Sheldrick, 1983; Hooper *et al.*, 1986; Moore *et al.*, 1986) and cattle (Walters *et al.*, 1984; Vighio & Liptrap, 1986) at the end of the oestrous cycle. Most of this oxytocin appears to be secreted from the CL (Walters *et al.*, 1984; Moore *et al.*, 1986). Flint & Sheldrick (1983) reported that 67% of episodes of PGFM release before luteolysis was complete were accompanied by the release of oxytocin. Synchronous episodes of oxytocin and $\text{PGF}_{2\alpha}$ secretion also occur following administration of a luteolytic dose of oestradiol at mid-cycle (Hixon & Flint, 1987). Uterine $\text{PGF}_{2\alpha}$ and luteal oxytocin comprise a positive feedback loop (Silvia *et al.*, 1991); oxytocin can stimulate uterine secretion of $\text{PGF}_{2\alpha}$, and $\text{PGF}_{2\alpha}$ can stimulate secretion of oxytocin from the CL. Pulsatile release of oxytocin is absent following ovariectomy (Flint & Sheldrick, 1983) and in hysterectomized cows bearing a persistent CL (Schams, Schallenberger, Meyer, Bullerman, Breiting, Enzenhofer, Koll, Kruip, Walters & Karg, 1985c). In sheep, synchronous episodes of oxytocin and $\text{PGF}_{2\alpha}$ secretion occur simultaneously in both left and right utero-ovarian veins (Hooper *et al.*, 1986) and $\text{PGF}_{2\alpha}$ administered into one uterine lymph vessel releases oxytocin in equal quantities from both ovaries (Heap *et al.*, 1989) when there is a CL on both sides. This may be due to recirculation of oxytocin (Flint *et al.*, 1990) or the involvement of a neural mechanism (Heap *et al.*, 1989) as

noradrenaline and acetylcholine stimulate oxytocin secretion *in vivo*. $\text{PGF}_2\alpha$ pulses preceded oxytocin pulses by about 17 minutes (Moore *et al.*, 1986) implying that activation of the feedback loop begins on the uterine side of the loop, but the stimulus initiating $\text{PGF}_2\alpha$ secretion is not known. Oxytocin from the posterior pituitary may be important during the initial phases of luteolysis (Hooper *et al.*, 1986; Lamsa & McCracken, 1986). In another study following a luteolytic dose of oestradiol, secretion of oxytocin by the CL was first detected 9 hours before detection of a luteolytic pulse of $\text{PGF}_2\alpha$ (Hixon & Flint, 1987). High concentrations of each hormone achieved within each pulse are a result of activation of this feedback loop (Flint, Sheldrick, Theodosis & Wooding, 1986).

Flint & Sheldrick (1983) suggested that each episode was terminated by the depletion of luteal stores of oxytocin. It is unlikely that the interval between pulses of $\text{PGF}_2\alpha$ is determined by the time required for the synthesis and replenishment of luteal oxytocin as proposed by Flint *et al.* (1986) because concentrations of mRNA for oxytocin in the CL are low during the luteolytic period (Ivell *et al.*, 1985; Jones & Flint, 1988). Transient uterine refractoriness to oxytocin could account for the time interval between pulses of $\text{PGF}_2\alpha$ (McCracken *et al.*, 1984). However, refractoriness is maintained for several days when oxytocin is administered chronically (Flint & Sheldrick, 1985; Kotwica, Schams, Meyer & Mittermeier, 1988; Gilbert, Lamming, Parkinson, Flint & Wathes, 1989; Howard, Morbeck & Britt, 1990; Lutz, Smith, Keisler & Garverick, 1990), but for only 6 hours after acute exposure (Sheldrick & Flint, 1986) and does not reflect down-regulation of the oxytocin receptor (Sheldrick & Flint, 1986). The ability of $\text{PGF}_2\alpha$ to stimulate luteal secretion of oxytocin is also suppressed after acute stimulation with $\text{PGF}_2\alpha$ (Watkins & Moore, 1987; Lamsa & McCracken, 1990).

The secretion of uterine $\text{PGF}_{2\alpha}$ is regulated by ovarian steroids. The uterus requires several days of exposure to progesterone in order to facilitate $\text{PGF}_{2\alpha}$ production during luteolysis (Woody *et al.*, 1967; Louis, Parry, Robinson, Thorburn & Challis, 1977; Ottobre *et al.*, 1980). Oxytocin can stimulate uterine secretion of $\text{PGF}_{2\alpha}$ in ovariectomized ewes or cows only after the animal has been exposed to progesterone for 7-10 days (Homanics & Silvia, 1988; Lafrance & Goff, 1988). Endometrial concentrations of lipid and eicosanoid precursors vary cyclically, being more abundant during progesterone-dominated phases of the oestrous cycle (Hansel *et al.*, 1975). The amount of prostaglandin H endoperoxide synthase (PGH synthase/cyclooxygenase) in uterine epithelial cells is increased when ovariectomized ewes are treated with progesterone (Raw, Curry & Silvia, 1988). Concentrations of cytoplasmic endometrial receptors for progesterone peak at oestrus and decline during the luteal phase of the oestrous cycle (Zelinski, Noel, Weber & Stormshak, 1982). Elevated concentrations of progesterone prevent the formation of receptors for oxytocin (Soloff, 1975). The rise in uterine oxytocin receptors at oestrus is closely linked to declining concentrations of progesterone (McCracken, 1980; Sheldrick & Flint, 1985; Fuchs *et al.*, 1990). The concentration of oxytocin receptors can be increased in ovariectomized ewes by treatment with progesterone for 10 days (Vallet, Lamming & Batten, 1990); this effect is not evident after five days treatment. Uterine secretion of $\text{PGF}_{2\alpha}$ can be stimulated by premature withdrawal of progesterone during the latter portion of the oestrous cycle (Kindahl, Lindell & Edqvist, 1981; Ottobre, Vincent, Silvia & Inskeep, 1984; Vincent & Inskeep, 1986). An increase in endometrial concentration of receptors for oxytocin can be detected within 6 hours of progesterone withdrawal in ewes (Leavitt, Okulicz, McCracken, Schramm & Robidoux, 1985). Lamming, Vallet & Flint (1991) and Flint, Stewart, Lamming & Payne (1992) conclude that the

effects of progesterone are dominant in the control of the concentration of oxytocin receptors which are crucial to the increase in $\text{PGF}_{2\alpha}$ release associated with luteal regression. After a period of exposure to biologically active concentrations of progesterone which inhibit the expression of oxytocin receptors, the uterus becomes refractory to progesterone and oxytocin receptors are increased. Both basal and oxytocin-induced activity of phospholipase C, a transmembrane signalling enzyme that may mediate the stimulatory effect of oxytocin on uterine prostaglandin secretion (Silvia & Homanics, 1988) are elevated after prolonged exposure to progesterone (Raw & Silvia, 1991).

Elevations in circulating concentrations of oestradiol coincide closely with the initial decline in progesterone concentrations (Nancarrow *et al.*, 1973; Baird *et al.*, 1976b; McCracken *et al.*, 1981; McCracken *et al.*, 1984). Blood flow to the uterus also increases at this time (Ford & Chenault, 1981). Increasing concentrations of oestrogen promote the formation of receptors for oestradiol (Clark, Pewck & Glasser, 1977) and oxytocin (Roberts, Barcikowski, Wilson, Skarnes & McCracken, 1975; Soloff, 1975; Roberts *et al.*, 1976; McCracken *et al.*, 1981; McCracken *et al.*, 1984; Hixon & Flint, 1987). Oestrogen treatment decreases lipid stores in the endometrium (Brinsfield & Hawk, 1973), increases oxytocin-induced phosphoinositide turnover (Hixon & Flint, 1987) and increases production of $\text{PGF}_{2\alpha}$ (Barcikowski *et al.*, 1974; Thatcher *et al.*, 1984; Knickerbocker *et al.*, 1986c). The ability of oxytocin to stimulate release of $\text{PGF}_{2\alpha}$ by uterine tissues from ovariectomized animals receiving progesterone is enhanced by treatment with oestrogen, either acutely (McCracken, 1980; Lafrance & Goff, 1988; Vallet *et al.*, 1990), or chronically (Homanics & Silvia, 1988; Raw & Silvia, 1991). In ovariectomized or seasonally anoestrous ewes oestrogen increases uterine responsiveness to oxytocin after 6 hours of administration (McCracken, 1980). This does not

appear to be true in the ovariectomized cow (Lafrance & Goff, 1988). However treatment of ovariectomized ewes with oestrogen has been shown to suppress concentrations of receptors for oxytocin (Vallet *et al.*, 1990). In cattle the amount of PGF_{2α} released in response to oxytocin, late in the oestrous cycle, is correlated with the relative concentration of oestrogen in the circulation at the time oxytocin is administered (Silvia & Taylor, 1989). The effects of oestrogen are much more pronounced when it is administered to animals that have previously been exposed to progesterone for 7 days or more (Silvia *et al.*, 1991).

1.10.Summary

While PGF_{2α} of uterine origin is generally accepted to be the endogenous agent responsible for the demise of the CL, many other factors, including other hormones, locally-produced ovarian substances and the presence of large antral follicles or a conceptus, can influence the lifespan of the CL. Furthermore, as a consequence of the wave pattern of follicular growth and development and the time interval between luteal regression and the onset of oestrus (which is dependent on the size of the largest non-atretic follicle present on the ovaries at the start of luteolysis), there is variability in the response of a group of animals to a prostaglandin oestrous synchronization programme apparently as a result of a random asynchrony of follicular development existing between animals, which can compromise the effectiveness of the treatment. Further studies are required to elucidate the relative importance of the different factors involved in the control of the lifespan of the CL, to permit more predictable synchronization between luteal regression, preovulatory follicular development and the onset of oestrus. The aim of these studies in heifers was to investigate the factor(s) which controls the formation, function and regression of the CL induced by the administration of GnRH in the early luteal phase of the oestrous cycle.

CHAPTER 2

MATERIALS AND METHODS

2.1.Experimental Animals

All animals used in the *in vivo* experimental studies were heifers between 12 months and 3 years of age. Two pools of animals were available: Hereford cross Friesian heifers purchased as calves and raised at the Institute farm at Blythbank, prior to transport to the Large Animal Unit at Dryden where the intensive experimental work was performed; and crossbred heifers of mixed breed (principally Angus cross, Simmentel cross, Blonde d'Aquitaine cross, Limousin cross and Charolais cross) raised at the University of Edinburgh's Easter Howgate farm and temporarily relocated to the Large Animal Unit for the duration of the trial. A "settling in" period was allowed between transport and the commencement of experiments to allow for acclimatisation and to confirm regular oestrous cyclicity. During the intensive phases of experimentation heifers were housed in covered courts on straw bedding. They were fed a diet of hay and grass nuts and water was available *ad libitum* with the exception of the 24 hour period preceding laparoscopy. Animals were weighed once a week to monitor body condition. All experiments were carried out within the Animals (Scientific Procedures) Act, 1986.

2.2.Surgical Procedures

2.2.1. Blood sampling

Daily blood samples, and blood samples taken during periods of more intensive "window" bleeds, were collected by jugular venepuncture. The animals were restrained in a conventional crush and haltered. Blood samples were allowed to clot overnight at room temperature and serum was harvested by aspiration of the supernatant following centrifugation at 1000 g for 30 minutes. Plasma samples, incorporating heparin as anticoagulant, were obtained similarly by aspiration of the supernatant following centrifugation at 1000 g for 30 minutes immediately after collection. Serum/plasma was stored at -20°C until required for hormone assay.

2.2.2. Laparoscopy

In preparation for laparoscopy, food was withheld for 48 hours and water was withheld for 24 hours. Animals were restrained in a tilted crush so the hindquarters were raised, and sedated with 2.5 mg/50 kg xylazine i.m. (Rompun: Bayer U.K. Ltd., Agrochem Division, Eastern Way, Bury St. Edmunds, Suffolk). Local anaesthesia was obtained by infiltration of 20 ml 2% lignocaine (Lignocaine: Univet 2 Ltd., Bicester, Oxon, UK). Two incisions were made in the right sublumbar fossa. A short probe was inserted into the upper incision to confirm penetration of the peritoneal cavity. Nitrous oxide was used to displace abdominal structures. When the reproductive tract had been located a metal guide was introduced into the lower incision and the short probe replaced by a long probe. The ovaries were located and all structures recorded. Following removal of the laparoscope, wounds were treated with oxytetracycline (Oxytetrin aerosol: Glaxovet Ltd., Breakspear Rd. South, Harefield, Uxbridge, Middlesex), and all heifers received 15 ml

benzathine penicillin/procaine penicillin (Duphaphen L.A.: Duphar Veterinary Ltd., Solvay House, Hedge End, Southampton).

2.3.Non-Surgical Procedures

2.3.1. Ultrasound examination

The ovaries of heifers were examined using a real time B-mode linear array ultrasound scanner (Aloka Echo Camera SSD-210 DX II) equipped with a 7.5 MHz intrarectal probe (UST-5511 I-7.5) (Aloka Co., Ltd., Japan). A plastic rod was attached to the cable of the probe to enable the manipulation of the probe from outside the rectum. Animals were restrained in a crush while the examination was carried out. The routine procedures for ultrasound scanning were as follows. Faeces were removed from the rectum and the right ovary was located and held in position with the left hand. The probe was inserted into the rectum and placed directly on top of the ovary held by the hand. The probe was manipulated from outside the rectum using the right hand to scan slowly over the top of the ovary several times. The procedure was repeated for the left ovary. Ultrasonography for each ovary was recorded on videotape (Kodak plc., USA) for subsequent analysis. Videotapes were reviewed using a TV monitor and the positions and sizes of all large and medium follicles and CL were recorded for each ovary.

2.4.Radioimmunoassays

Unless otherwise stated all reagents were from Sigma Chemical Co. (Poole, Dorset, UK).

2.4.1. Assay buffers

Assay buffers routinely used in the laboratory were freshly prepared for each assay, using water purified by reverse osmosis through a Waters

Milli-Q purification system (Millipore Corporation, Milford, MA, USA) as diluent. All buffers were filtered through filter paper (Whatman Qualitative No. 1, W. & R. Balston Ltd.) to remove particulate matter. Bacterial contamination was prevented by the incorporation of thimerosal.

(1) *0.9% saline in 0.05M phosphate (PBS)*

This buffer contained 9 g of sodium chloride, 100 ml of phosphate buffer (0.5M sodium dihydrogen orthophosphate and 0.5M disodium hydrogen orthophosphate) and 100 mg thimerosal per litre.

(2) *0.1% gelatin/PBS buffer (0.1%-gelatin)*

This buffer contained 9 g of sodium chloride, 1 g of swine skin gelatin (300 bloom), 100 ml of phosphate buffer and 100 mg thimerosal per litre. The gelatin was dissolved in 200 ml of buffer at 50°C prior to final dilution.

(3) *0.1% BSA/PBS buffer (0.1%-BSA)*

This buffer contained 9 g sodium chloride, 1 g bovine serum albumin (BSA: Fraction V) (Boehringer Mannheim GmbH, Germany), 100 ml phosphate buffer and 100 mg thimerosal per litre.

2.4.2. Assay calculations

All assays were analysed on a Macintosh computer using the AssayZap software (Zaristow Software, Haddington, East Lothian, Scotland). The assay sensitivities, and inter- and intra-assay coefficients of variation are detailed in the relevant chapters.

2.4.3. Luteinizing hormone

Peripheral serum LH concentrations were measured by the double antibody RIA described by Price *et al.*, (1987). The preparation used for iodination was USDA-bLH-I1 (iodinated by the method of Greenwood, Hunter & Glover, 1963) and the hormone preparation used as standard was USDA-bLH-B5. Standard solutions (ranging from 0.05 to 2.0 ng/tube) in triplicate, and serum samples (100 μ l) in duplicate, were made up to 500 μ l in 12 x 75 mm plastic tubes (LIP Equipment and Services Ltd., West Yorks, England) with assay buffer (0.1%-BSA). Specific antibody (R.B. Staigmiller, USDA, ARS, Montana Agric. Exp. Stn., Miles City, MT 59301) raised in rabbits and affinity-purified on cyanogen bromide-activated sepharose-4B, was diluted in assay buffer and added (200 μ l/tube) at an initial dilution of 1:20,000. The assay was incubated at 4°C for 48 hours. Then tracer (approximately 12,000 cpm [¹²⁵I]-LH in 100 μ l) was added and the tubes incubated for a further 48 hours at 4°C. The antibody-bound LH and free LH were separated by the addition of 100 μ l of normal rabbit serum at an initial dilution of 1:400, and 200 μ l of donkey anti-rabbit serum (NRS and DARS: both obtained from Scottish Antibody Production Unit (SAPU), Carlisle) at an initial dilution of 1:45 incorporating 10% 0.1M ethylenediaminetetraacetic acid (EDTA) in assay buffer, followed by incubation overnight at 4°C. Following the addition of 1 ml cold assay buffer, the tubes were centrifuged at 2000 g for 30 minutes at 4°C. The supernatant was decanted, the tubes allowed to drain, the rims aspirated and the activity in the pellet counted on a gamma counter (1277 Gammamaster, Pharmacia Wallac, Milton Keynes, UK).

2.4.4. Follicle-stimulating hormone

Peripheral serum FSH concentrations were measured by a RIA routinely used in our laboratory (Bolt & Rollins, 1983; Law *et al.*, 1992). The preparation used for iodination was USDA-bFSH-BP3 (iodinated by the lactoperoxidase method of Thorrel & Johansson, 1971) and the hormone preparation used as standard was USDA-bFSH-B-1. Standard solutions (ranging from 0.5 to 32 ng/tube) in triplicate, and serum samples (250 µl) in duplicate, were made up to 500 µl in 12 x 75 mm plastic tubes with assay buffer (0.1%-BSA). Specific antibody (USDA-5-POOL: bFSH-B antiserum) was diluted in assay buffer and added (200µl/tube) at an initial dilution of 1:10,000. The assay was incubated at 4°C for 48 hours. Then tracer (approximately 12,000 cpm [¹²⁵I]-FSH in 100µl) was added and the tubes incubated for a further 48 hours at 4°C. The antibody-bound FSH and free FSH were separated by the addition of 100 µl of NRS at an initial dilution of 1:400, and 200 µl of DARS at an initial dilution of 1:45 incorporating 10% 0.1M EDTA in assay buffer, followed by incubation overnight at 4°C. Following the addition of 1 ml cold assay buffer, the tubes were centrifuged at 2000 g for 30 minutes at 4°C. The supernatant was decanted, the tubes allowed to drain, the rims aspirated and the activity in the pellet counted on a gamma counter.

2.4.5. Oestradiol

2.4.5.1. Serum oestradiol

Peripheral serum oestradiol concentrations were measured by a RIA incorporating an affinity chromatography extraction as described by Webb, Baxter, McBride, Nordblom & Shaw (1985).

Oestradiol antibody (sheep anti-oestradiol (pool) 26/9/80) was coupled to cyanogen bromide-activated sepharose-4B as follows. Sepharose-4B (9 g) was placed on a grade 3 sintered filter funnel and washed with 2 litres 1 mM hydrochloric acid in 200 ml aliquots. The slurry was then immediately added to 150 μ l sheep anti-oestradiol antiserum/coupling buffer (0.1M sodium bicarbonate buffer, pH 8.3 containing 0.5M sodium chloride) in a ground-glass stoppered tube and mixed overnight on an end-over-end mixer at 4°C. The mixture was filtered on a sintered glass filter funnel (porosity 1), and an excess (65 ml) of 1M ethanolamine (pH 9.0) was added to the slurry to block remaining active groups and mixed for a further 2 hours at room temperature. Ethanolamine was removed from the antibody-linked sepharose on the sintered funnel with alternate washes of acetate buffer (0.1M sodium acetate buffer, pH 4.0 containing 0.5M sodium chloride) and coupling buffer (300 ml/wash) over 3 cycles to remove non-covalently adsorbed protein. Finally the antibody-linked sepharose was resuspended in 70 ml water containing 0.01% thimerosal. A binding check was carried out to optimise the volume of antibody-linked sepharose to be used in the assay.

For extraction, approximately 1500 counts per 5 min (2,4,6,7,16,17- ^3H)-oestradiol-17 β (Amersham International plc, UK) in 10 μ l HPLC-grade absolute ethanol (FSA Laboratory Supplies, Loughborough) to allow estimation of recovery efficiency, was dispensed into 16 x 125 mm screw-capped glass culture tubes, followed by 3 ml serum sample. The tubes were vortexed and incubated at room temperature for 30 minutes. Milli-Q water (7 ml) and 400 μ l sepharose-linked ovine anti-oestradiol antiserum were then added, the tubes capped and mixed end-over-end overnight.

Chromatography columns (10 x 120 mm soda glass) fitted with porosity 1 sinter discs (Schott Glass, UK) were washed with 1 x 3 ml 90% methanol

(Distol reagent: FSA Laboratory Supplies, Loughborough) followed by 3 x 10 ml Milli-Q water. This cycle was repeated 3 times. The contents of the extraction tubes were poured directly onto the columns and allowed to drain to waste, the tubes rinsed with 10 ml Milli-Q water and the washings subsequently added to the columns. Columns were then washed 3 times with 7 ml Milli-Q water. Excess moisture retained on the columns after the last washing was removed by applying gentle air pressure, and the antibody-bound extracted oestradiol eluted with 3 ml 90% methanol into 16 x 125 mm glass tubes. Again positive pressure was applied to ensure maximum elution. Methanol was evaporated under vacuum in a vortex evaporator (Haake/Buchler) at 40°C, and the extracted steroid reconstituted into 1.8 ml 0.1%-gelatin followed by a further mixing for 30 minutes at 40°C. Reconstituted sample (500 µl) was removed into plastic scintillation vials and counted in a beta-counter (1410 Liquid Scintillation Counter, Pharmacia Wallac, Milton Keynes, UK) for [³H] activity. Estimates of total activity added and the level of background activity were also obtained and the recovery efficiency determined. Reconstituted samples (2 x 500 µl) were taken for assay. The sepharose-linked ovine anti-oestradiol was then recovered, recycled (washing with 1 x 3 ml 90% methanol and 3 x 7 ml Milli-Q water over 3 cycles), made up to the original volume with water and kept at 4°C for re-use. Chromatography columns were also recycled by washing with 3 cycles of 1 x 3 ml 90% methanol and 3 x 7 ml Milli-Q water before re-use. All glassware (except columns) was washed in the dishwasher and baked at 200°C for 2 hours.

The assay procedure was as follows. Standards (in triplicate) ranging from 0.5 to 48 pg/tube in 500 µl 0.1%-gelatin, and reconstituted samples, were incubated with 200 µl antiserum (R48: initial dilution 1:40,000, raised in rabbits against oestradiol-11β-succinyl bovine serum albumin) and tracer

(approximately 12,000 cpm [^{125}I]-17 β -oestradiol-11 α -tyrosinemethylester in 100 μl , iodinated by the method of Webb, Baxter, McBride, Nordblom & Shaw, 1985) in 12 x 75 mm glass assay tubes (LIP Equipment and Services Ltd., West Yorks, England) for 2 hours at room temperature. The antibody-bound oestradiol and free oestradiol were separated by the addition of 100 μl each of NRS (SAPU) at an initial dilution of 1:400 in 0.1%-gelatin and DARS (SAPU) at an initial dilution of 1:40 in 0.1%-gelatin incorporating 10% 0.1M EDTA in assay buffer, followed by incubation overnight at 4°C. Following the addition of 1 ml cold assay buffer, the tubes were centrifuged at 2000 g for 30 minutes at 4°C. The supernatant was decanted, the tubes allowed to drain, the rims aspirated and the activity in the pellet counted on a gamma counter.

In each assay water blanks were included to estimate the amount of interference due to water and glassware contamination. Control samples from cattle and sheep in known physiological states which covered a wide range of oestradiol concentrations were also included. The quantity of oestradiol added as recovery label was assayed to allow for correction. When used at an initial dilution of 1:40,000 the antibody-bound 30-50% of radio-labelled tracer added.

2.4.5.2. Luteal oestradiol

(1) *Extraction validation*

Preliminary studies were carried out to determine whether it was necessary to follow the steroid extraction with affinity chromatography. Ovaries were collected from the slaughterhouse on ice, CL decapsulated and weighed and washed with PBS. Luteal tissue (400 mg) was removed for double extraction (solvent extraction followed by affinity chromatography). PBS (1 ml/100 mg luteal tissue) was added in round-based plastic centrifuge tubes. Homogenization on ice (2 x 15 second bursts) with a Polytron (X-1020:

International Laboratory Apparatus, GmbH, Germany) was followed by centrifugation at 1000 g for 30 minutes. The supernatant was removed and stored at -20°C until double extraction.

For solvent extraction, approximately 70,000 counts per 5 min (2,4,6,7,16,17-[³H])-oestradiol-17β in 100 μl HPLC-grade absolute ethanol was dispensed into 16 x 125 mm screw-capped glass culture tubes, to allow estimation of recovery efficiency, followed by 2 ml supernatant in duplicate (A and B). 100 pg of oestradiol (10 μl of 10 ng/ml oestradiol in ethanol) was added to A. The tubes were vortexed and incubated at room temperature for 30 minutes. 10 ml of a mixture of 4 parts hexane:1 part diethyl ether (both from FSA Laboratory Supplies, Loughborough) (i.e. solvent:aqueous ratio of 5:1) was added, the tubes vortexed for 30 minutes and then allowed to stand for 5 minutes to allow a clear interface to form. The aqueous layer was frozen (60 minutes at -80°C). The solvent layer was decanted into glass tubes and evaporated under vacuum in a vortex evaporator without heat, and the extracted steroid reconstituted into 3 ml PBS, followed by a further mixing for 30 minutes with heat (40°C). 100 μl was removed for estimation of recovery following steroid extraction. The extracted steroid was then split into 2 aliquots. The first aliquot was subjected to affinity chromatography. Aliquots (100, 200 and 400 μl) of the sample were removed and made up to 3 ml with PBS. Following the affinity chromatography procedure (described above in section 2.4.5.1.), 500 μl was removed for estimation of recovery following affinity chromatography, and duplicates of 500 μl were removed for RIA. Duplicates of 100, 200 and 400 μl of the second aliquot were removed directly for RIA. The RIA procedure was as described above (section 2.4.5.1.).

Water blanks and control samples were included as described above (section 2.4.5.1.). The quantities of oestradiol added as recovery label were assayed to allow for subtraction of tritiated oestradiol added to unknowns.

Oestradiol added to luteal samples was recovered with an accuracy of $115.4 \pm 25.8 \%$. The results obtained from the double extraction procedure were comparable to the results obtained when solvent extraction alone was used.

(2) *Final procedure*

Ovaries were processed as described above. Luteal tissue (300 mg) was removed for solvent extraction. Approximately 7000 counts per 5 min (2,4,6,7,16,17- ^3H)-oestradiol-17 β in 10 μl HPLC-grade absolute ethanol was dispensed into 16 x 125 mm screw-capped glass culture tubes, followed by 1 ml supernatant in duplicate. The tubes were vortexed and incubated at room temperature for 30 minutes. 5 ml of a mixture of 4 parts hexane:1 part diethyl ether was added, the tubes vortexed for 30 minutes and then allowed to stand for 5 minutes. The aqueous layer was frozen (60 minutes at -80°C). The solvent layer was decanted into glass tubes and evaporated under vacuum in a vortex evaporator without heat, and the extracted steroid reconstituted into 1 ml PBS, followed by a further mixing for 30 minutes with heat. 100 μl was removed for estimation of extraction efficiency. Duplicates of 100, 200 and 300 μl of the extract were removed directly for RIA (section 2.4.5.1.).

2.4.6. Progesterone

2.4.6.1. Serum progesterone

Peripheral serum progesterone concentrations were measured by a RIA described by Corrie, Hunter & Macpherson (1981) and modified for a non-extraction procedure (Law *et al.*, 1992). The preparation used for iodination was progesterone-11 α -glucuronide-tyramine (iodinated by the method of Corrie, Ratcliffe & Macpherson (1982) and supplied by the MRC

Centre for Reproductive Biology, Edinburgh; or obtained from Amersham International plc, Amersham, UK). Standard solutions (ranging from 7.8 to 1000 pg/tube in triplicate, to which 50 μ l ovariectomized heifer serum was added) and serum samples (50 μ l in duplicate) were made up to 500 μ l in 12 x 75 mm glass tubes with assay buffer (0.1%-gelatin). Standards and samples were incubated with 200 μ l antiserum (R31/8: initial dilution 1:16,000, raised in rabbits against progesterone-11 α -hemisuccinate bovine serum albumin) and tracer (approximately 12,000 cpm [125 I]-progesterone in 100 μ l, incorporating 1 mg/ml 8-anilino-1-naphthalene sulfonic acid (ANS) to prevent the interference of steroid-binding proteins) in glass assay tubes (12 x 75 mm) for 3 hours at room temperature. The antibody-bound progesterone and free progesterone were separated by the addition of 100 μ l each of NRS at an initial dilution of 1:300 in 0.1%-gelatin and DARS at an initial dilution of 1:35 in 0.1%-gelatin incorporating 10% 0.1M EDTA in assay buffer, followed by incubation overnight at 4°C. Following the addition of 1 ml cold assay buffer, the tubes were centrifuged at 2000 g for 30 minutes at 4°C. The supernatant was decanted, the tubes allowed to drain, the rims aspirated and the activity in the pellet counted on a gamma counter. When used at an initial dilution of 1:16,000 the antibody-bound 35-55% of radio-labelled tracer added.

2.4.6.2. Luteal progesterone

(1) *Extraction validation*

Preliminary studies were carried out to optimise the volume and type of extracting solvent, the effect of heat on the evaporation process, the volume of the reconstituting medium, the effect of heat on the reconstitution process, and the recovery volume. In addition steroid residues in the aqueous phase and in the glass tube following reconstitution were

determined. For solvent extraction, approximately 5000 counts per 5 min (1,2,6,7,16,17- ^3H)-progesterone (Amersham International plc, UK) in 200 μl HPLC-grade absolute ethanol was dispensed into large (20 x 150 mm) or small (15 x 100 mm) screw-capped glass culture tubes, to allow estimation of recovery efficiency, followed by 300 μl (large tubes only) water. The tubes were vortexed and incubated at room temperature for 30 minutes. Petroleum ether (5 ml or 2 ml) (Distol reagent: FSA Laboratory Supplies, Loughborough or Analar reagent: BDH, Poole, England), or 5 ml of a mixture of 4 parts hexane:1 part diethyl ether (i.e. solvent:aqueous ratio of 10:1) was added, the tubes vortexed for 30 minutes and then allowed to stand for 5 minutes to allow a clear interface to form. The aqueous layer was frozen by standing for 60 minutes at -80°C . The solvent layer was decanted into glass tubes and evaporated under vacuum in a vortex evaporator with heat (30°C) or without heat, and the extracted steroid reconstituted into 0.5, 1.0, 1.5, or 2.0 ml 0.1%-gelatin or 4 ml scintillation fluid (SF; Optiphase X: Pharmacia Fine Chemicals, Uppsala, Sweden), followed by a further mixing for 30 minutes with heat (40°C or 60°C). SF containing steroid was taken directly for recovery estimation. 100 μl 0.1%-gelatin containing steroid was removed for recovery estimation. Steroid residues in the aqueous phase were determined by taking 100 μl aqueous phase for recovery estimation, and in the glass tube from the difference in percentage recovery from the SF (which would remove all steroid residues remaining on the glass tube) and from 0.1%-gelatin following reconstitution.

Table 2.1. Comparison of 2 solvents with/without heat in the evaporation process (without heat in the reconstitution process).

SOLVENT		% STEROID IN AQUEOUS RESIDUE	RECONSTITUTING MEDIUM		HEAT	% RECOVERY
Petroleum ether	Hexane/ diethyl ether		SF	0.1%- gelatin		
5 ml	-	25	4 ml	-	+	56
5 ml	-	26	-	0.5 ml	+	31
5 ml	-	21	4 ml	-	-	53
5 ml	-	27	-	0.5 ml	-	31
2 ml	-	58	4 ml	-	+	21
2 ml	-	61	-	0.5 ml	+	19
2 ml	-	71	4 ml	-	-	25
2 ml	-	72	-	0.5 ml	-	14
-	5 ml	18	4 ml	-	-	64
-	5 ml	18	-	0.5 ml	-	46

Table 2.2. Comparison of different volumes of reconstituting medium with heat (40°C or 60°C) in the reconstitution process (5 ml petroleum ether as solvent, without heat in the evaporation process).

0.1%-GELATIN	HEAT	% RECOVERY
0.5 ml	40°C	38
1.0 ml	40°C	43
1.5 ml	40°C	46
2.0 ml	40°C	47
0.5 ml	60°C	42
1.0 ml	60°C	47
1.5 ml	60°C	50
2.0 ml	60°C	51

Table 2.3. Comparison of 2 types of petroleum ether (5 ml volume of solvent, without heat in the evaporation process, 2 ml reconstitution volume, with heat (60°C) in the reconstitution process).

PETROLEUM ETHER	% RECOVERY
FSA	55
BDH	59

Percentage recovery was greatest with 5 ml volume of extracting solvent, with hexane:diethyl ether when compared to petroleum ether (however, petroleum ether was used in the final procedure because of increased laboratory availability), with heat (60°C) in the reconstitution process, and with increasing reconstitution volume. Some steroid remained in the aqueous phase and on the side of the glass tube. Heat (30°C) in the evaporation process did not influence the percentage recovery.

(2) *Final procedure*

Ovaries were collected from the slaughterhouse on ice, CL decapsulated and weighed and washed with PBS. Luteal tissue (300 mg) was removed for solvent extraction. PBS (1 ml/100 mg luteal tissue) was added to round-bottomed, plastic centrifuge tubes. Homogenization on ice (2 x 15 second bursts with a polytron) was followed by centrifugation at 1000 g for 30 minutes. The supernatant was removed and stored at -20°C until extraction.

For solvent extraction, approximately 2000 counts per 5 min (1,2,6,7,16,17-[³H])-progesterone in 100 µl HPLC-grade absolute ethanol was dispensed into 16 x 125 mm screw-capped glass culture tubes, to allow estimation of recovery efficiency, followed by 50µl supernatant and 350 µl

PBS. The tubes were vortexed and incubated at room temperature for 30 minutes. Petroleum ether (5 ml; i.e. solvent:aqueous ratio of 10:1) was added, the tubes vortexed for 30 minutes and then allowed to stand for 5 minutes to allow a clear interface to form. The aqueous layer was frozen by standing for 60 minutes at -80°C. The solvent layer was decanted into glass tubes and evaporated under vacuum in a vortex evaporator with heat (30°C), and the extracted steroid reconstituted into 2 ml PBS, followed by a further mixing for 30 minutes with heat (60°C). 500 µl was removed for recovery estimation. 40 µl extract was removed and diluted to 1 ml with PBS. Duplicates of 100 µl of the diluted extract were removed directly for RIA.

Progesterone concentrations in luteal extracts were measured by a RIA similar to that described above (section 2.4.6.1.), omitting the addition of ovariectomized heifer serum to the standards, and omitting the addition of ANS to the tracer. Standard solutions (ranging from 10 to 1000 pg/tube in triplicate), and diluted extract samples (100 µl in duplicate), were made up to 500 µl with assay buffer (0.1%-gelatin).

Water blanks and control samples were included, and the quantity of progesterone added as recovery label was assayed to allow for correction.

2.4.6.3. Media progesterone

Media progesterone concentrations were measured by a RIA similar to that described above (section 2.4.6.1.), omitting the addition of ovariectomized heifer serum to the standards, and omitting the addition of ANS to the tracer. Standard solutions (ranging from 10 to 1000 pg/tube in triplicate), and diluted media samples (100 µl in duplicate), were made up to 500 µl with assay buffer (0.1%-gelatin).

2.4.7. Oxytocin

2.4.7.1. Luteal oxytocin

(1) *Radioimmunoassay validation*

Reagents are detailed in the final procedure.

(a) Binding check with [^{125}I]-oxytocin fractions.

Following chloramine-T iodination of synthetic oxytocin, the reaction solution was separated by gel filtration chromatography on a Sephadex G25 column with 0.1%-BSA as elution buffer. Fractions of 1 ml [^{125}I]-oxytocin were collected and a binding check carried out on fractions 7, 8, 9 and 10, from the descending portion of the second radioactive peak. Fractions were diluted in 0.1%-BSA (approximately 12,000 cpm [^{125}I]-oxytocin in 50 μl) and incubated overnight at 4°C with 50 μl oxytocin antiserum at an initial dilution of 1:300, followed by addition of 50 μl 1:400 NSS and 50 μl 1:30 DASS containing 1:10 0.1M EDTA and incubation overnight at 4°C. Binding of label to antibody was maximal (56%) with fraction 10.

(b) Binding check with varying concentrations of antiserum.

[^{125}I]-oxytocin was diluted in 0.1%-BSA (approximately 12,000 cpm in 50 μl) and incubated overnight at 4°C with 50 μl oxytocin antiserum at initial dilutions of 1:5000, 1:10,000, 1:20,000, and 1:40,000, followed by addition of 50 μl 1:300 or 1:400 NSS and 50 μl 1:30 or 1:40 DASS containing 1:10 0.1M EDTA and incubation

overnight at 4°C. Binding of label to antibody was optimal (30%) with oxytocin antiserum at an initial dilution of 1:20,000, 1:300 NSS and 1:30 DASS containing 1:10 0.1M EDTA (Figure 2.1.).

(c) Optimisation of assay volume.

The assay was reoptimised for an increased initial reaction volume of 500 µl to increase accuracy when dispensing reagents on a large scale. Following extrapolation of the lines shown in Figure 2.2. binding of label to antibody was optimal (30%) with oxytocin antiserum at an initial dilution of between 1:80,000 and 1:100,000 with 100 µl (1:300) and 200 µl DASS (1:30) containing 1:10 0.1M EDTA. For all subsequent assays the initial dilution of the oxytocin antiserum was 1:100,000 to maximise use of available antibody.

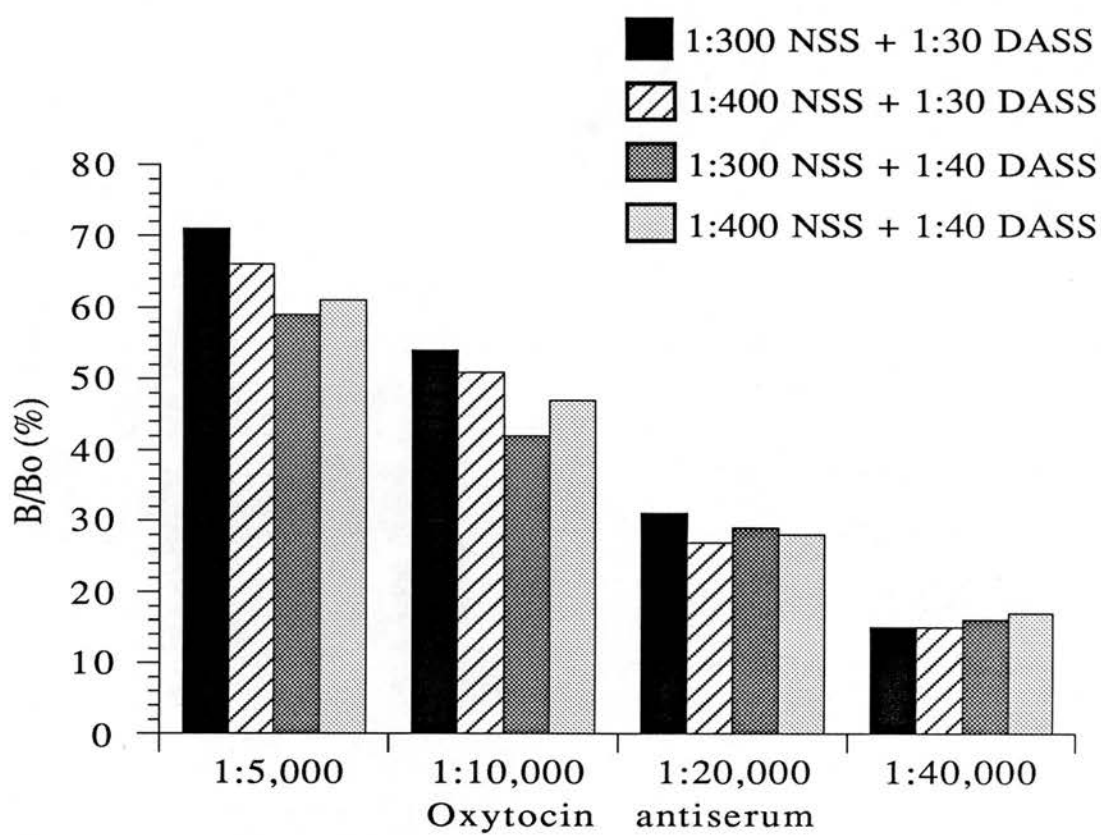
(d) Assay specificity.

Cross-reactivities of various bovine pituitary hormones (calculated as the ratio of oxytocin standard required to suppress binding of radio-labelled oxytocin to 50% of maximum binding to the amount of hormone required to produce a similar effect), were assessed as follows:- arginine vasopressin, arginine vasotocin: <0.005%.

(e) Quality controls.

Plasma samples of known oxytocin concentration (donated by D.C. Wathes, IAPGR, Cambridge Research Station) were extracted by the method of Wathes, Ayad, Gilbert, McGoff & Wathes (1991) for use as quality controls in the assay.

Figure 2.1. Binding (%) of [^{125}I]-oxytocin to 4 concentrations of oxytocin antiserum. The antibody-bound and free oxytocin were separated by the addition of varying concentrations of normal sheep serum (NSS) and donkey anti-sheep serum (DASS).



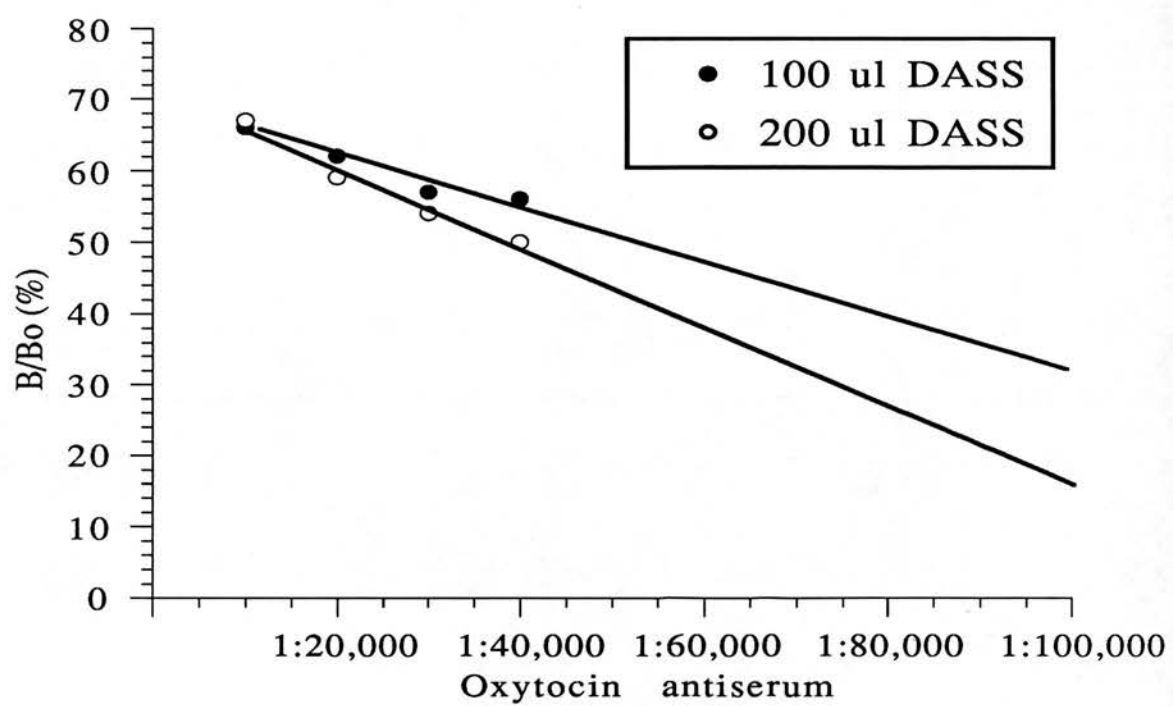


Figure 2.2. Binding (%) of [125 I]-oxytocin to 4 concentrations of oxytocin antiserum. The antibody-bound and free oxytocin were separated by the addition of 1:300 normal sheep serum (NSS) and either 100 μ l or 200 μ l of 1:30 donkey anti-sheep serum (DASS).

(2) *Final procedure*

Oxytocin was extracted from the CL by a method described by Tsang, Walton & Hansel (1990). Ovaries were collected from the slaughterhouse on ice, CL decapsulated and weighed and washed with PBS. Luteal tissue (300 mg) was removed for extraction. Acetic acid (1 ml 1% acid/100 mg luteal tissue) was added to round-bottomed, plastic centrifuge tubes, followed by homogenization on ice (2 x 15 second bursts with a polytron). Approximately 8000 counts per 5 min [^3H]-oxytocin (NEN, UK) in 10 μl HPLC-grade absolute ethanol was added to allow estimation of recovery efficiency, and the tubes vortexed. The homogenate was centrifuged in polycarbonate microcentrifuge tubes at 11,000 g for 30 minutes at 4°C. The supernatant was decanted into microcentrifuge tubes and frozen overnight at -80°C. The supernatant was thawed and recentrifuged at 11,000 g for 30 minutes at 4°C. 100 μl was removed for recovery estimation. Aliquots of extract (10 μl) were removed and diluted to 1 ml with 0.1%-BSA. Duplicates of the diluted extract (100 μl) were removed directly for RIA.

Oxytocin concentrations in luteal extract were measured by a RIA developed for routine use in our laboratory. The hormone preparation used for iodination (iodinated by the chloramine-T method of Greenwood *et al.*, 1963) and as standard, was synthetic oxytocin (Cambridge Research Biochemicals, UK) dissolved in 0.2% acetic acid at a concentration of 1 mg/ml. Standard solutions (ranging from 0.25 to 500 pg/tube in triplicate), and extract samples (100 μl in duplicate), were made up to 500 μl in 12 x 75 mm plastic tubes with assay buffer (0.1%-BSA). Specific antibody (GJ242: oxytocin antiserum; MRC Centre for Reproductive Biology, Edinburgh) was diluted in assay buffer and added (200 μl /tube) at an initial dilution of 1:100,000. The assay was incubated at 4°C overnight. Then tracer

(approximately 12,000 cpm [125 I]-oxytocin in 100 μ l) was added and the tubes incubated at 4°C overnight. The antibody-bound oxytocin and free oxytocin were separated by the addition of 100 μ l of normal sheep serum (NSS; SAPU) at an initial dilution of 1:300, and 200 μ l of donkey anti-sheep serum (DASS; SAPU) at an initial dilution of 1:30 incorporating 10% 0.1M EDTA in assay buffer, followed by incubation overnight at 4°C. Following the addition of 1 ml cold assay buffer, the tubes were centrifuged at 2000 g for 30 minutes at 4°C. The supernatant was decanted, the tubes allowed to drain, the rims aspirated and the activity in the pellet counted on a gamma counter.

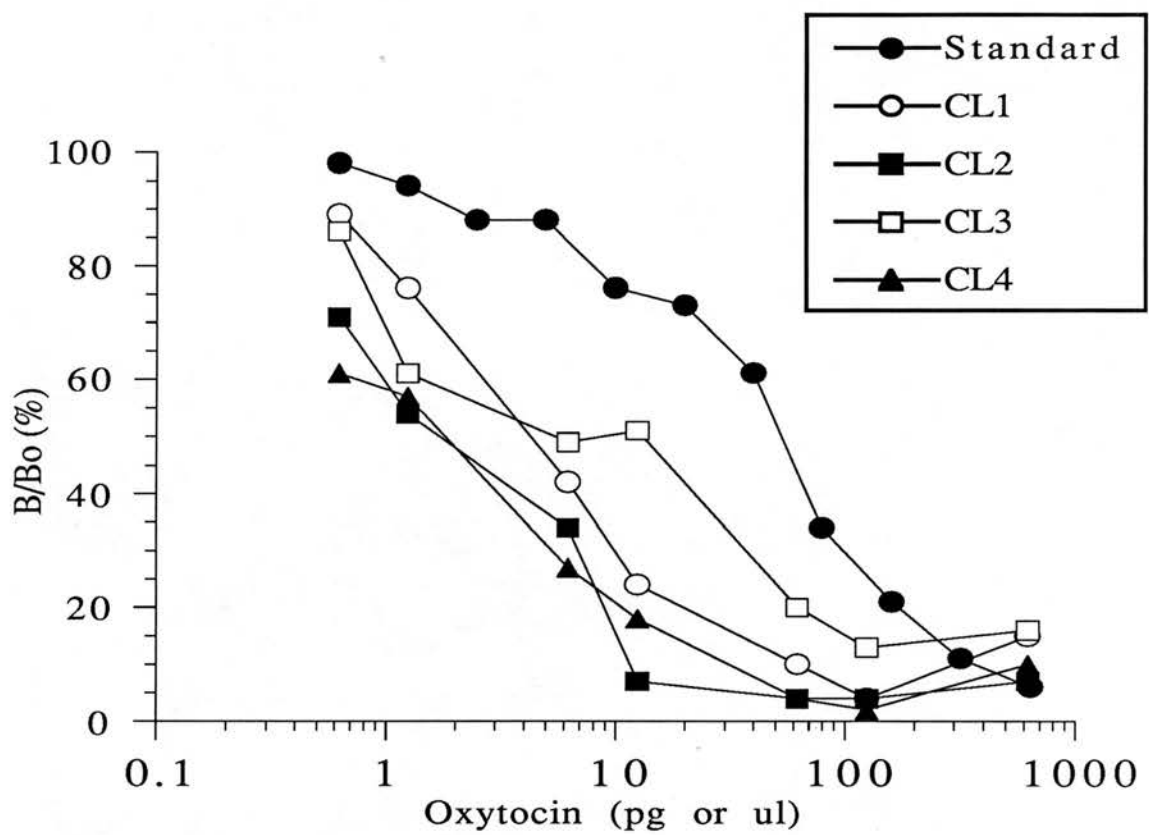
The quantity of oxytocin added as recovery label was assayed to allow for correction. When used at an initial dilution of 1:100,000 the antibody-bound 23-29% of radio-labelled tracer added.

Between the ED80 and the ED20 concentrations, the dilution curves of extracted luteal tissue from 4 CL obtained from a local abattoir were parallel to the standard curve (Figure 2.3.).

2.4.8. 13,14-dihydro-15-keto-prostaglandin F_{2 α}

Peripheral plasma PGFM concentrations were measured by a RIA developed by Dr. R. Kelly, MRC Centre for Reproductive Biology, Edinburgh. The preparation used for iodination was 13,14-dihydro-15-keto-prostaglandin F_{2 α} coupled to proline-tyrosine (iodinated by the chloramine-T method of Greenwood *et al.*, 1963) and the hormone preparation used as standard was PGFM. Standard solutions (ranging from 1 to 1000 pg/ml) in quadruplicate, and plasma samples (50 μ l) in quadruplicate, were made up to 300 μ l with assay buffer (0.1%-BSA-40mM Tris-HCl). Specific antibody to PGFM was raised in sheep against a conjugate of PGFM coupled to Keyhole Limpet Haemocyanin, and purified by affinity chromatography. Antibody was diluted in assay buffer and added (100 μ l/tube) at an initial dilution of

Figure 2.3. Comparison of a standard curve for oxytocin and dilution curves of extracted luteal tissue from 4 bovine CL obtained from a local abattoir.



1:10,000. Tracer (approximately 25,000 cpm [^{125}I]-PGFM in 100 μl) was added, the tubes vortexed and incubated at 4°C for 16 hours. The antibody-bound PGFM and free PGFM were separated by the addition of 0.5 ml 0.25% bovine gamma globulin and 1 ml 25% polyethylene glycol as precipitant. The tubes were vortexed and centrifuged at 2500 g for 30 minutes at 4°C. The supernatant was aspirated and the activity in the pellet counted on a gamma counter.

Standard curves and unknowns were also run with:

- (a) Boiled serum (3 minutes).
- (b) Trichloroacetic acid-treated serum (to precipitate serum proteins).
- (c) Incubation with protease inhibitors (to demonstrate that inhibition of binding of radio-labelled PGFM was not due to degradation of the antibody and/or tracer by proteases).
 - (i) Phenylmethylsulfonyl fluoride (PMSF-dissolved in ethanol: serine protease inhibitor: 2mM).
 - (ii) N-Ethylmaleimide (thiol protease inhibitor: 2 mM).
 - (iii) Pepstatin A (carboxypeptidase inhibitor: 1 $\mu\text{g/ml}$).
 - (iv) EDTA (metalloprotease inhibitor: 2 mM).
 - (v) All 4 inhibitors together.
 - (vi) Ethanol (control for PMSF).

Treatments (a)-(c) did not affect the activity of the plasma samples or the standard curve. However, extraction of plasma with organic solvent

(ethyl acetate) markedly reduced plasma activity (<15%). PGFM concentrations in plasma samples incorporating heparin were not significantly different from PGFM concentrations in plasma samples incorporating EDTA and aspirin.

2.5.Receptor Assays

2.5.1. Luteinizing hormone

LH binding to bovine CL was determined as described by Bramley, Stirling, Swanston, Menzies & Baird (1987). The characteristics for binding were identical to those described for LH binding to the human CL (personal communication, Dr T.A.Bramley).

2.6.DNA Assay

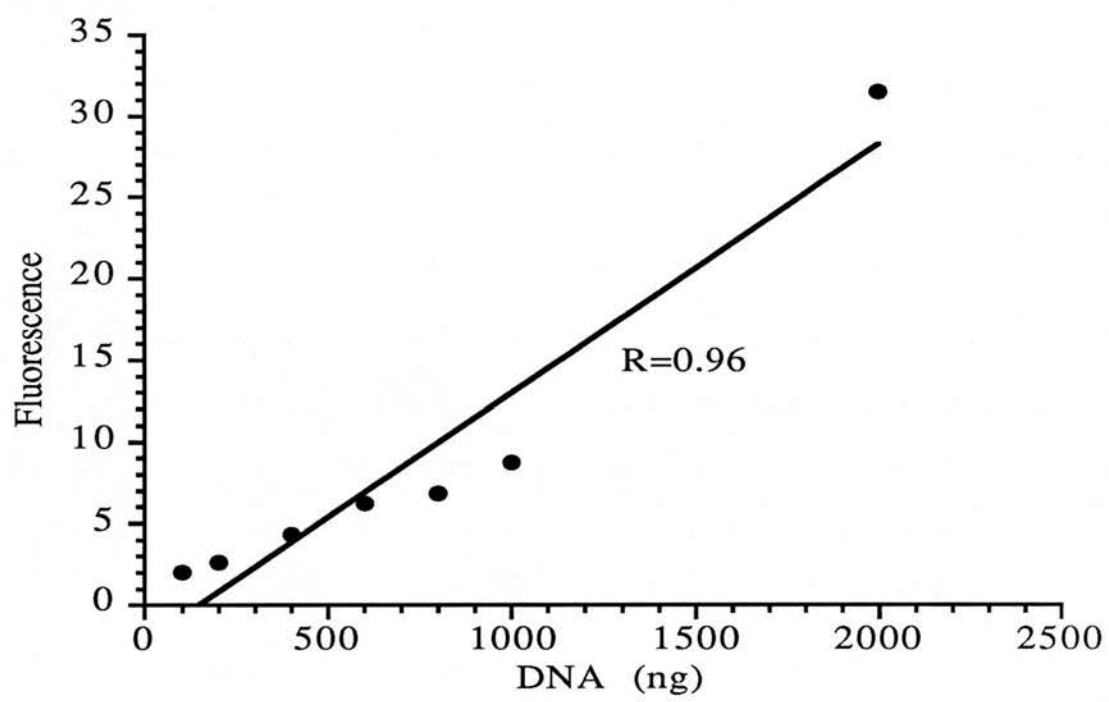
To determine the number of cultured luteal cells/well, deoxyribonucleic acid (DNA) contents were measured by the method of West, Scattar & Kumar (1985). An example of a DNA standard curve is shown in Figure 2.4.

2.7.Follicular Fluid

2.7.1. Processing of bFF for in vivo studies

Bovine follicular fluid was collected by aspiration of medium (5-10 mm diameter) and large (>10 mm diameter) follicles visible on the surface of the ovaries which had been obtained from a local slaughterhouse. The follicular fluid was stored at -20°C. All bFF collections were thawed, filtered and pooled for extraction of steroids. Follicular fluid was stirred overnight with dextran-coated charcoal (DCC: 1 mg/ml activated charcoal; Hopkins and Williams, Chadwell Heath, Essex, England, and 0.1 mg/ml T70 dextran; Pharmacia Fine Chemicals, Uppsala, Sweden). These concentrations of

Figure 2.4. The DNA standard curve used to determine DNA content to monitor cell number following the study of the effect of bFF fractions on progesterone production by cultured bovine luteal cells (see Chapter 7, section 7.2.4.). Samples measured for fluorescence in a Perkin-Elmer Fluorescence Spectrophotometer (Excitation=385 nm; Emission=450 nm).



charcoal and dextran have been shown to remove most follicular fluid steroids without affecting inhibin activity (Tsonis, Quigg, Lee, Leversha, Trounson & Findlay, 1983). The follicular fluid was then centrifuged at 4000 g for 30 minutes at 4°C, and refrozen in 100 ml aliquots at -20°C. The procedure removed approximately 98% of oestradiol and progesterone.

2.7.2. Processing of bFF for in vitro studies

Bovine follicular fluid was collected and prepared as described above (section 2.7.1.) and then subjected to affinity chromatography on red sepharose followed by anion exchange chromatography on QAE for the separation of "inhibin-like" proteins from the bFF (Law, 1991).

Steroid-stripped bFF (50 ml) was mixed with approximately 400 ml red sepharose CL-6B (Pharmacia Fine Chemicals, Uppsala, Sweden) overnight at 4°C on an end-over-end mixer. The "inhibin-like" proteins bound to the sepharose beads. The mixture was then separated on a sintered glass funnel (porosity 4), connected to a water vacuum pump. The sepharose beads were then washed with 1 litre of equilibration buffer (1 litre 0.1M Tris mixed with 0.1M HCl until pH 7.6, supplemented with 480 g urea plus 1 litre 0.7M KCl [= 0.57 mMho. conductivity]) followed by 700 ml elution buffer (1 litre 0.1M Tris mixed with 0.1M HCl until pH 7.6, supplemented with 480 g urea plus 1 litre 2.4M KCl [= 1.2 mMho. conductivity]). The unbound fraction (R1) and the bound fraction (R2) were dialysed against PBS and then concentrated by Amicon filtration (stirred cell apparatus with YM5 [5000 MW cut-off] membrane) to remove high salt concentrations.

After red sepharose separation the 2 bFF fractions (R1: unbound to sepharose; and R2: bound to sepharose) were further fractionated by anion exchange chromatography on QAE sephadex. Each fraction was mixed with 200 ml QAE exchanger resin, equilibrated in 20mM Tris/HCl buffer pH 7.6

containing 0.5M urea with 0.1M NaCl, at 4°C overnight. The QAE slurry treated with R1 or R2 was then batch-separated in a Pharmacia column connected to an LKB Uvicord S detector type 2138, by application of a step gradient of increasing salt concentration in the 20mM Tris-HCl-urea-NaCl buffer system pH 7.6. The protein peaks were collected and concentrated and dialysed on the Amicon system as before. Finally each peak was dialysed in Visking tubing against 0.15M PBS buffer, pH 7.5 (several changes of PBS over 2 days at 4°C). Each fraction was then filtered using Sartorius Minisart NML microfilters (0.2 µm cut-off).

Total protein was assessed in each fraction by the method of Lowry, Rosebrough, Farr & Randall (1951), by testing several dilutions of the unknown preparation against known concentrations of a BSA standard, and reading their absorbance at 620/690 nm on a Titertek Multiscan micro plate reader.

2.8. Dispersion of Bovine Luteal Cells

For *in vitro* studies, bovine luteal cells were obtained by collagenase dispersion of luteal tissue using a procedure developed in our laboratory from the methods of Simmons *et al.*, (1976) and Hixon & Hansel (1979), following preliminary studies to optimise experimental conditions as described in Chapter 7 (see section 7.2.1.). The detailed procedures were as follows.

- (1) *Preparation of enzyme solution:* Medium 199 (5 ml/g luteal tissue; Gibco Life Technologies Ltd., Paisley, Scotland) containing antibiotics (0.1 g/l penicillin-G and 0.1 g/l streptomycin sulphate) and sodium bicarbonate (1.1 g/l), was supplemented with 20mM Hepes (N'-2-hydroxyethylpiperazine-N'-

ethanesulphonic acid), 1% BSA, type V collagenase (2000 U/g luteal tissue) and DNase (1 mg/g luteal tissue).

- (2) *Preparation of post-lysing buffer solution:* HBSS (Gibco Life Technologies Ltd., Paisley, Scotland) was diluted 1:10 with double-distilled water.
- (3) *Preparation of post-dispersion solution:* Medium 199 containing antibiotics and sodium bicarbonate, was supplemented with 20mM Hepes and 1% BSA.
- (4) *Preparation of luteal tissue:* Bovine ovaries were collected from a local abattoir into Medium 199 containing antibiotics and sodium bicarbonate (holding medium) and transported to the laboratory at 37°C in a thermos-flask within 1 hour of slaughter. The CL were decapsulated and weighed and divided into 1 g pieces. Each piece was weighed and minced in holding medium. The suspension was centrifuged at 100 g for 2-3 minutes and the supernatant removed with a Pasteur pipette. The tissue fragments were incubated with enzyme solution (5 ml/g luteal tissue) for 2.5 hours in a shaking bath (120 cycles/minute) at 37°C. The cell suspension and undigested CL were passed through a sterile 100 µm filter. The cell suspension was centrifuged at 100g for 5 minutes and the supernatant removed with a Pasteur pipette. Red Cell Lysing Buffer (1 ml) was added to the pellet and mixed gently for 1 minute to resuspend the cells. 10 ml post-lysing buffer solution was added to the suspension, centrifuged at 100 g for 5 minutes and the supernatant removed with a Pasteur pipette. The cell pellet was washed 3 times with 5 ml post-dispersion solution. Following the final centrifugation the cell

pellet was resuspended in 5 ml post-dispersion solution and all suspensions from a single CL were combined. Cell number was estimated using a haemocytometer. Cell viability was assessed by trypan blue exclusion (cell suspensions were diluted 1:1 with 0.4% trypan blue solution and incubated for 10-15 minutes: dead cells stained blue) and was >95%.

2.9.Separation of Bovine Luteal Cells

Separation of dispersed bovine luteal cells into large and small cell fractions was carried out using a Beckman JE-6B elutriator rotor equipped with a Sanderson chamber, following a preliminary study to optimise experimental conditions as described in Chapter 7 (see section 7.2.2.). The detailed procedures were as follows. After collagenase dispersion of luteal tissue (see section 2.8.) the cell suspension was centrifuged at 100 g for 5 minutes, the supernatant removed with a Pasteur pipette and the cell pellet resuspended in elutriating buffer (PBS, incorporating 1% (w/v) BSA which coats the tubing and prevents cell adhesion). The cell suspension was loaded onto the elutriator in 5 ml aliquots. From each aliquot, approximately 30 x 10 ml fractions were obtained (the number of fractions depended on the flow rate of the peristaltic pump, the volume of the eluting buffer and the volume of the collecting vial). Each fraction was centrifuged at 100g for 5 minutes, supernatants were removed with a Pasteur pipette and the cell pellets resuspended in 0.5-2 ml post-elutriation solution (Medium 199 containing antibiotics and sodium bicarbonate, supplemented with 20mM Hepes and 1% BSA). A haemocytometer was used to estimate cell number per fraction, from which the total number of elutriated cells was calculated. Cell viability was assessed by trypan blue exclusion and was >95%. A micrometer eyepiece was used to classify elutriated cells as small luteal cells ($\geq 10 \mu\text{m}$ and $< 25 \mu\text{m}$ diameter) or large luteal cells ($\geq 25 \mu\text{m}$ diameter). The result of an initial

study to compare the ratio of small to large luteal cells pre-elutriation with the ratio post-elutriation (in the total luteal cells recovered from all fractions), is shown in Table 2.4.

Table 2.4. Percentage of small and large luteal cells before and after elutriation.

	SMALL LUTEAL CELLS (%)	LARGE LUTEAL CELLS (%)
PRE-ELUTRIATION	92.0	8.0
POST-ELUTRIATION	92.1	7.9

2.10.Culture of Bovine Luteal Cells

Bovine luteal cells were cultured using an ovine granulosa cell serum-free culture system developed in our laboratory (Webb & McBride, 1991) with slight modifications. The procedure was as follows.

- (1) *Coating of plates:* 500 μ l of donor calf serum (Gibco Life Technologies Ltd., Paisley, Scotland) was added to each of the 24 central wells of the plates (48-well tissue culture clusters: Costar, MA, USA). The peripheral wells were not used because they can be affected by evaporation. The plates were incubated overnight at 37°C in an atmosphere of 2% carbon dioxide and 98% air.
- (2) *Culture medium:* Medium 199 (Gibco Life Technologies Ltd., Paisley, Scotland) containing antibiotics (0.1 g/l penicillin-G and 0.1 g/l streptomycin sulphate), Earle's salts and Hepes (25mM).
- (3) *Plating out:* The donor calf serum was decanted from the precoated plates and the plates were washed 3 times with 500 μ l

culture medium. Luteal cells were then plated out at a density of 0.5×10^6 cells/ml (250 μ l/well). The plates were incubated for 24 hours at 37°C in an atmosphere of 2% carbon dioxide and 98% air to allow the cells to adhere to the plate. It was important not to disturb the plates as movement may prevent cell attachment.

- (4) *DNA measurement*: Culture plates were stored at -40°C. The density of luteal cells was monitored by DNA assay (see section 2.6.).

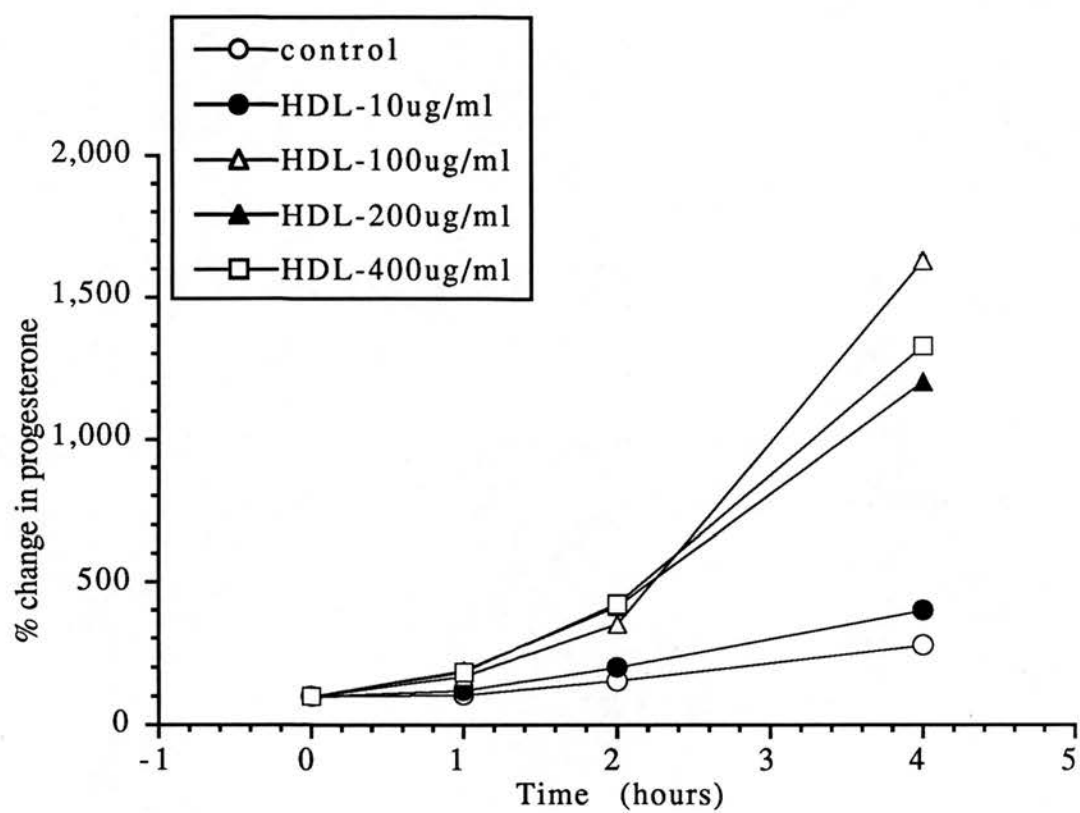
2.11. Incubation of Bovine Luteal Cells

2.11.1. Short-term incubation of dispersed luteal cells

A preliminary study was carried out to optimise the concentration of HDL cholesterol to be included in the incubation medium. Incubation media were prepared with 0, 10, 100, 200 and 400 μ g/ml HDL cholesterol (Lipoproteins: Sigma Chemical Co., Poole, Dorset, UK, containing 9.2 mg/ml total cholesterol, which was 90.7% HDL cholesterol). After a preincubation period (30 minutes) dispersed bovine luteal cells were incubated for 4 hours and aliquots of medium (containing cells) removed at 0, 1, 2, and 4 hours for progesterone assay as described below. The dose-response curve is shown in Figure 2.5. Following the results of the preliminary study, HDL cholesterol was incorporated into the incubation medium at a concentration of 200 μ g/ml.

A second preliminary study was carried out to optimise the concentration of dbcAMP ($N^6,2'$ -O-dibutyryladenosine-3':5'-cyclic monophosphate; sodium salt) with which to challenge the luteal cells. After a preincubation period, dispersed bovine luteal cells were incubated for 4 hours with 1, 0.5, 0.25, 0.125, and 0.0625 mg dbcAMP per ml, and aliquots of

Figure 2.5. Response of dispersed bovine luteal cells to 0, 10, 100, 200 and 400 $\mu\text{g/ml}$ HDL cholesterol over a 4-hour incubation period. Progesterone production is expressed as a percentage of progesterone production in the preincubation period.

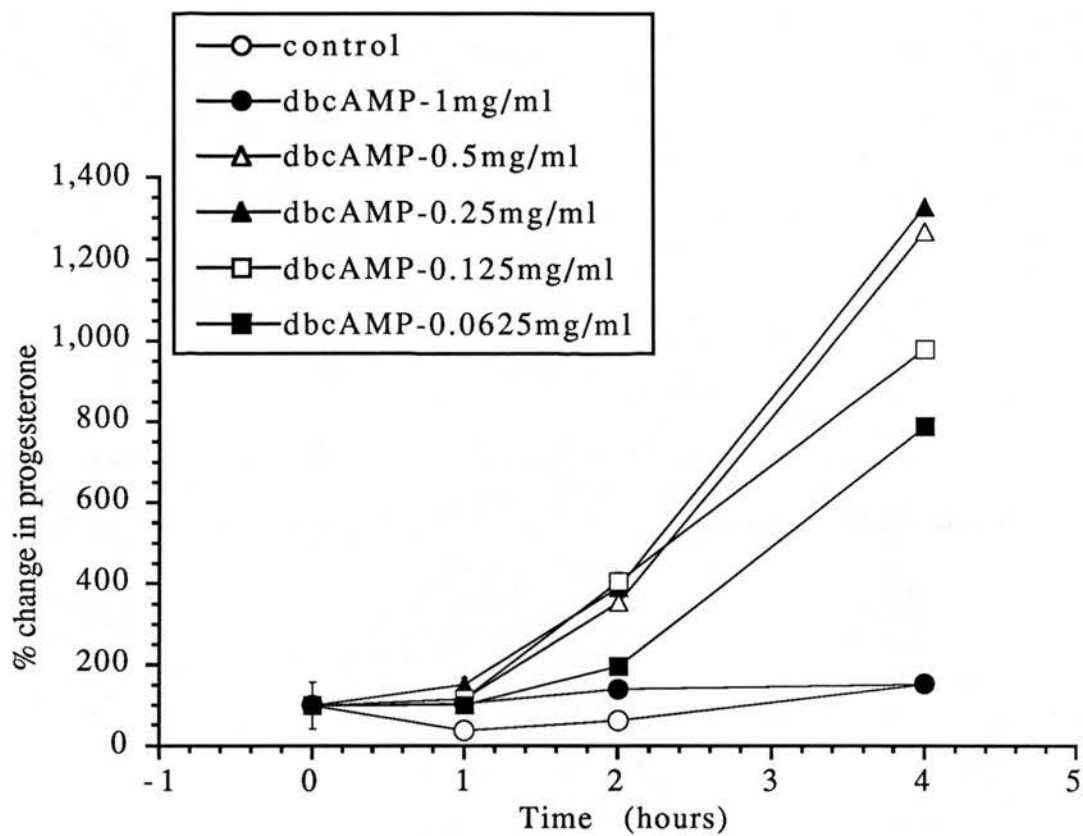


medium plus cells removed at 0, 1, 2, and 4 hours for progesterone assay as described above. The dose-response curve is shown in Figure 2.6. Following the results of the preliminary study, cells were challenged with 0.5 mg/ml dbcAMP (see Chapter 8, section 8.2.4.).

For short-term incubations of dispersed luteal cells, bovine luteal cells were obtained by collagenase dispersion of luteal tissue as described in section 2.8. After assessment of cell number and cell viability, the cell suspension was centrifuged at 100 g for 5 minutes, the supernatant removed with a Pasteur pipette and the cell pellet resuspended in incubation medium to achieve a final concentration of $0.5-1.0 \times 10^6$ viable cells/ml. The procedure was as follows.

- (1) *Preparation of incubation medium:* Medium 199 (5 ml/g luteal tissue; Gibco Life Technologies Ltd., Paisley, Scotland) containing antibiotics and sodium bicarbonate was supplemented with 20mM Hepes and 200 µg/ml HDL cholesterol.
- (2) *Incubation of luteal cells:* Cell suspensions were dispensed to 12 x 75 mm glass tubes (2 ml/tube) and incubated for 30 minutes (preincubation) in a shaking bath (60 cycles/minute) at 37°C. At the end of the preincubation period a 0.5 ml aliquot (representing incubation medium and cells) was removed and the incubation stopped by freezing.
- (3) *Treatments:* At the end of the preincubation period the cells were challenged with various treatments as detailed in Chapter 8 (3 replicates/treatment) and incubated in the shaking bath (60 cycles/minute) at 37°C. 0.5 ml aliquots were removed at 1, 2 and 4 hours and the incubation stopped by freezing.

Figure 2.6. Response of dispersed bovine luteal cells to 0, 1, 0.5, 0.25, 0.125 and 0.0625 mg/ml dbcAMP over a 4-hour incubation period. Progesterone production is expressed as a percentage of progesterone production in the preincubation period.



- (4) *Progesterone production*: Media progesterone concentrations were determined by direct (unextracted) RIA after freeze-thawing to disrupt the cells.

2.11.2.Short-term incubation of cultured luteal cells

For short-term incubations of cultured luteal cells, bovine luteal cells were obtained by collagenase dispersion of luteal tissue as described in section 2.8. After assessment of cell number and cell viability, the cell suspension was centrifuged at 100 g for 5 minutes, the supernatant removed with a Pasteur pipette and the cell pellet resuspended in culture medium (Medium 199 containing antibiotics, Earle's salts and Hepes), to achieve a final concentration of 0.5×10^6 viable cells/ml. The procedure was as follows.

- (1) *Preparation of culture plates*: Culture plates were prepared as described in section 2.10.
- (2) *Culture of luteal cells*: Cell suspensions were dispensed into the 24 central wells of a 48-well culture plate (250 μ l: 125,000 cells/well) and incubated overnight at 37°C in an atmosphere of 2% carbon dioxide and 98% air. At the end of the plating-down period medium was removed from each well and stored at -40°C.
- (3) *Treatments*: At the end of the plating-down period the cells were challenged with various treatments as detailed in Chapter 7 (3 replicates/treatment) and incubated at 37°C in an atmosphere of 2% carbon dioxide and 98% air. After 4 hours medium was removed from each well and stored at -40°C.

- (4) *DNA measurement*: Culture plates were stored at -40°C. Cell density/well was determined by DNA assay.
- (5) *Progesterone production*: Media progesterone concentrations were determined by direct (unextracted) RIA.

2.12. Statistical Analyses

All data are presented as arithmetic means \pm sem. Statistical differences between treatment groups were tested using ANOVA and Student's *t* test, allowing for repeated measurements if applicable. Specific details relevant to the particular data sets are described in the appropriate chapters. In Chapter 8 linear models were fitted to the hormone profiles. Hormone profiles were compared by linear regression analysis using the Genstat package (Rothamstead Experimental Station).

CHAPTER 3

INVESTIGATION OF THE EFFECT OF GnRH IN THE EARLY LUTEAL PHASE OF THE OESTROUS CYCLE OF HEIFERS

3.1.Introduction

Three waves of follicular development were observed in the majority of bovine oestrous cycles, although 2-wave and 4-wave patterns have been identified (see Chapter 1, section 1.4). It is likely that the number of waves per cycle is determined by the length of the cycle rather than being an intrinsic characteristic of the cycle. However it is only the large antral follicle that is present on the ovary at the onset of luteal regression in the late luteal phase which escapes atresia and undergoes further rapid development to the preovulatory stage. Dominant follicles of the first and second waves are destined to become atretic by an as yet unknown mechanism.

An experiment was carried out to investigate the relationships between follicular and luteal function. The objectives of the experiment were firstly, to investigate whether the dominant follicle could ovulate in response to a single injection of GnRH in the early luteal phase, and secondly, to determine whether the induced CL was functional, following

PGF_{2α} treatment to remove the endogenous CL. Day 6 was selected as the day of GnRH administration, since it has been demonstrated previously that the highest response (83%) to an ovulatory dose of hCG was achieved between Days 4 and 7 of the oestrous cycle (Price & Webb, 1989). GnRH was used rather than hCG to achieve a more physiological LH surge profile.

3.2. Materials and Methods

3.2.1. Animals

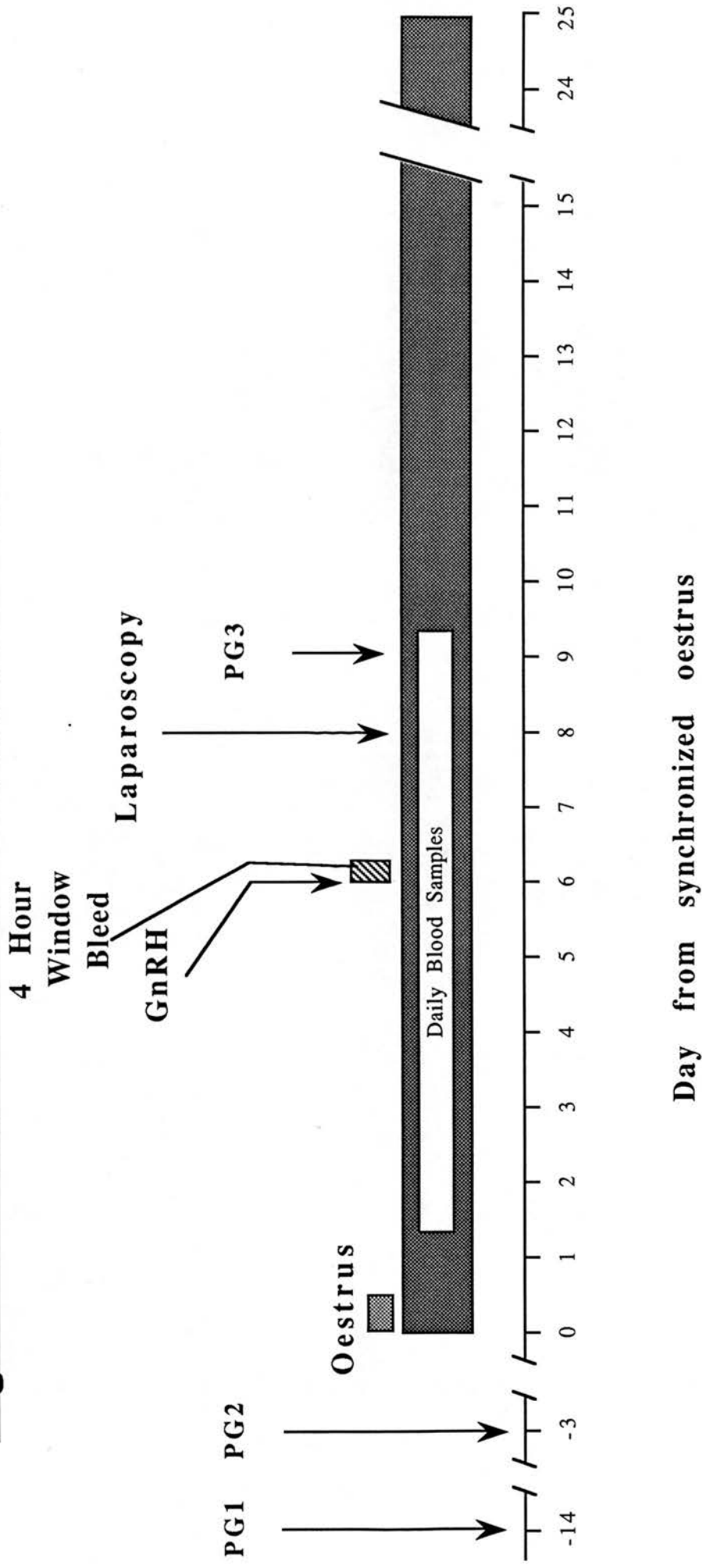
Mature Hereford cross Friesian heifers were housed in straw yards on the Institute Large Animal Unit and fed a maintenance ration of hay and grass nuts.

3.2.2. Experimental procedure

The experimental protocol is shown in Figure 3.1. The oestrous cycles of 19 heifers were synchronized with 2 injections of 500 µg cloprostenol (Estrumate: Coopers Animal Health, Crewe Hall, Cheshire), 11 days apart (PG1 and PG2). Oestrous behaviour following PG2 was monitored by 3 times-daily observation, together with the use of heat detection devices (Kamar Heatmount Detectors: Kamar Inc., Steamboat Springs, CO, USA). Oestrus was confirmed retrospectively by daily blood sampling for serum progesterone concentrations. Six days after the synchronized oestrus (oestrus = Day 0) all heifers received 0.5 mg gonadorelin (GnRH; Fertagyl: Intervet Laboratories Ltd., Science Park, Milton Rd., Cambridge) by i.m. injection, which was followed by a 4-hour window bleed to characterise the LH response.

In preparation for laparoscopy, food was withheld for 48 hours and water was withheld for 24 hours. All heifers were subjected to sublumbar laparoscopy 2 days after the gonadorelin injection. Animals were sedated with 2.5 mg/50 kg xylazine (Rompun: Bayer U.K. Ltd., Agrochem Division,

Figure 3.1. PROTOCOL FOR EXPERIMENT



Eastern Way, Bury St. Edmunds, Suffolk) i.m., and local anaesthesia was obtained by infiltration of 20 ml 2% lignocaine (Lignocaine: Univet 2 Ltd., Bicester, Oxon, UK). All visible structures on both ovaries were recorded. Wounds were treated with oxytetracycline (Oxytetrin aerosol: Glaxovet Ltd., Breakspear Rd. South, Harefield, Uxbridge, Middlesex), and all heifers received 15 ml benzathine penicillin/procaine penicillin (Duphaphen L.A.: Duphar Veterinary Ltd., Solvay House, Hedge End, Southampton).

Animals with no new ovulation point were classified as non-responders. Heifers with a fresh ovulation point, and hence a new induced CL, were classified as GnRH responders (subsequent cycle referred to as the induced cycle, where day of GnRH injection = Day 0). Twenty-four hours after laparoscopy and 3 days after GnRH administration, all heifers received 500 µg cloprostenol (PG3) at a time when the spontaneously-formed CL would be in the PGF_{2α}-responsive phase of the cycle and the induced CL would be expected to be non-responsive. Oestrous behaviour was recorded as before; blood sampling frequency was increased to 3 times-daily for 3 days to closely monitor the serum progesterone concentrations, and then continued once-daily until all heifers had shown oestrus. Animals were monitored by rectal ultrasonography once-daily, using a real-time B-mode linear array ultrasound scanner (Aloka Echo Camera SSD-210 DX II) equipped with a 7.5 MHz intra-rectal probe (UST-5511 I-7.5) (Aloka Co. Ltd., Japan), preceding GnRH administration, to record follicular status, and from 5 days after GnRH to confirm the presence or absence of the induced CL and follow the growth of the largest follicle.

3.2.3. Hormone radioimmunoassays

Blood samples were collected by jugular venepuncture, and serum harvested and stored at -20°C for subsequent measurement of peripheral progesterone and LH concentrations.

3.2.3.1. Progesterone assay

Samples from this study were assayed for progesterone in one assay. The sensitivity of the assay was 8.2 pg/tube (0.2 ng/ml) and the intra-assay coefficient of variation was 10.9%.

3.2.3.2. LH assay

Samples from this study were assayed for LH in one assay. The sensitivity of the assay was 50.0 pg/tube (0.5 ng/ml) and the intra-assay coefficient of variation was 11.7%.

3.2.4. Statistical analysis

All data are presented as means \pm sem. Differences between the GnRH responders and non-responders in the interval from PG3 to oestrus were tested by Student's *t* test. Progesterone profiles were analyzed on a daily basis in the period following PG3, when mean serum progesterone concentration first fell below 1 ng/ml, to the sample preceding the mean day of oestrus for the GnRH responders, and compared to the profile of the non-responders over the same time period. Differences between total progesterone secreted were tested using Student's *t* test. LH profiles were analyzed following GnRH during the LH surge, and the total amount of hormone secreted was determined from the area under the peak.

3.3.Results

3.3.1. Ovarian observations

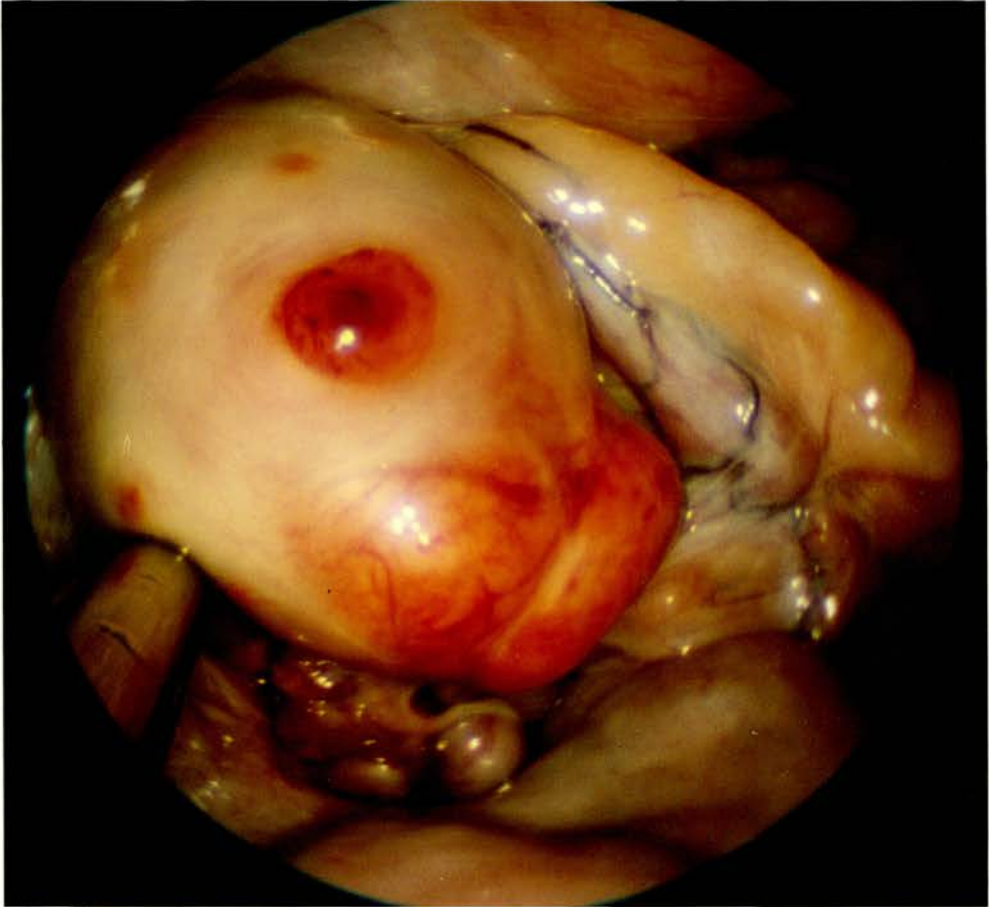
Identification of luteal structures on the ovary at laparoscopy corresponded to the classification of Ireland, Murphree & Coulson (1980). Thus new ovulation points on Day 1 of the induced cycle appeared red, small (<1 cm diameter) and were not covered by epithelium. Spontaneously-formed mid-cycle corpora lutea were brown, tan or orange, larger (>1.5 cm diameter) and epithelialised with some peripheral surface vasculature. Figure 3.2. shows the ovulation point of a GnRH responder.

Ultrasound observations confirmed the emergence of a large follicle from the first wave of follicular growth in the oestrous cycle following the synchronized oestrus. There were no apparent differences between responders and non-responders in the characteristics (size and growth rate) of the large follicle before GnRH treatment, similar to previously published observations (Gong, Bramley & Webb, 1993). Ultrasonography from 5 days after GnRH confirmed the presence of an induced CL in the GnRH responders, and followed the emergence of a new wave of follicular growth which coincided approximately with the emergence of the second (post-synchronized oestrus) wave of follicles in the GnRH responders.

3.3.2. Response to GnRH

Fourteen (74%) of the 19 heifers responded to the GnRH injection with an additional ovulation. The mean interval from PG3 to oestrus was extended by 4.7 days ($P<0.01$) in the GnRH responders compared to the non-responders.

Figure 3.2. Photograph at laparoscopy of the ovary of a GnRH responder showing a large, orange-yellow, spontaneously formed CL and a small, red, new, GnRH-induced ovulation point. (Magnification x4.0)



3.3.3. Peripheral LH concentrations

Mean serum LH profiles for the non-responders and GnRH responders are shown in Figure 3.3. All heifers had a surge release of LH following GnRH. There was no significant difference in the magnitude of the LH surge between non-responders (11.8 ± 3.4 ng/ml) and responders (12.9 ± 1.8 ng/ml), or in the total amount of hormone secreted.

3.3.4. Peripheral progesterone concentrations

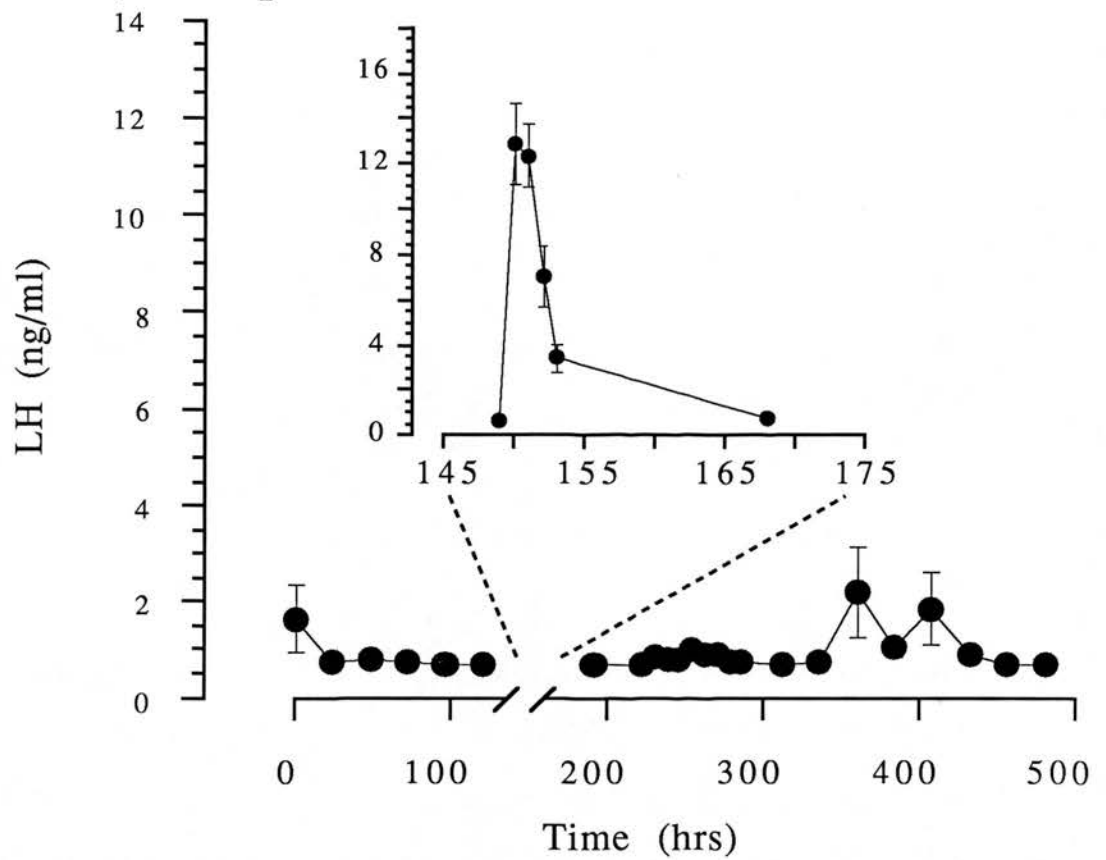
Mean serum progesterone profiles for the non-responders and GnRH responders are shown in Figure 3.4. Around the time of GnRH administration there were marked fluctuations in progesterone concentrations presumably associated with the LH stimulation of the spontaneously-formed luteal tissue. Following PG3 mean serum progesterone concentrations fell in both the non-responders and the GnRH responders. However, the delay in regression of the induced CL was associated with a significantly elevated mean progesterone profile in the GnRH responders ($P < 0.01$).

3.4. Discussion

These studies have shown for the first time that administration of GnRH to heifers in the early luteal phase of the oestrous cycle results in ovulation and the formation of an additional CL in approximately 75% of animals. This demonstrates that the dominant follicle of the first wave is competent to ovulate and form a functional CL in response to a surge release of LH.

The reason for the 25% non-response rate is not known but agrees with the previously published results of Price & Webb (1989) using hCG. The

a) Responders



b) Non-Responders

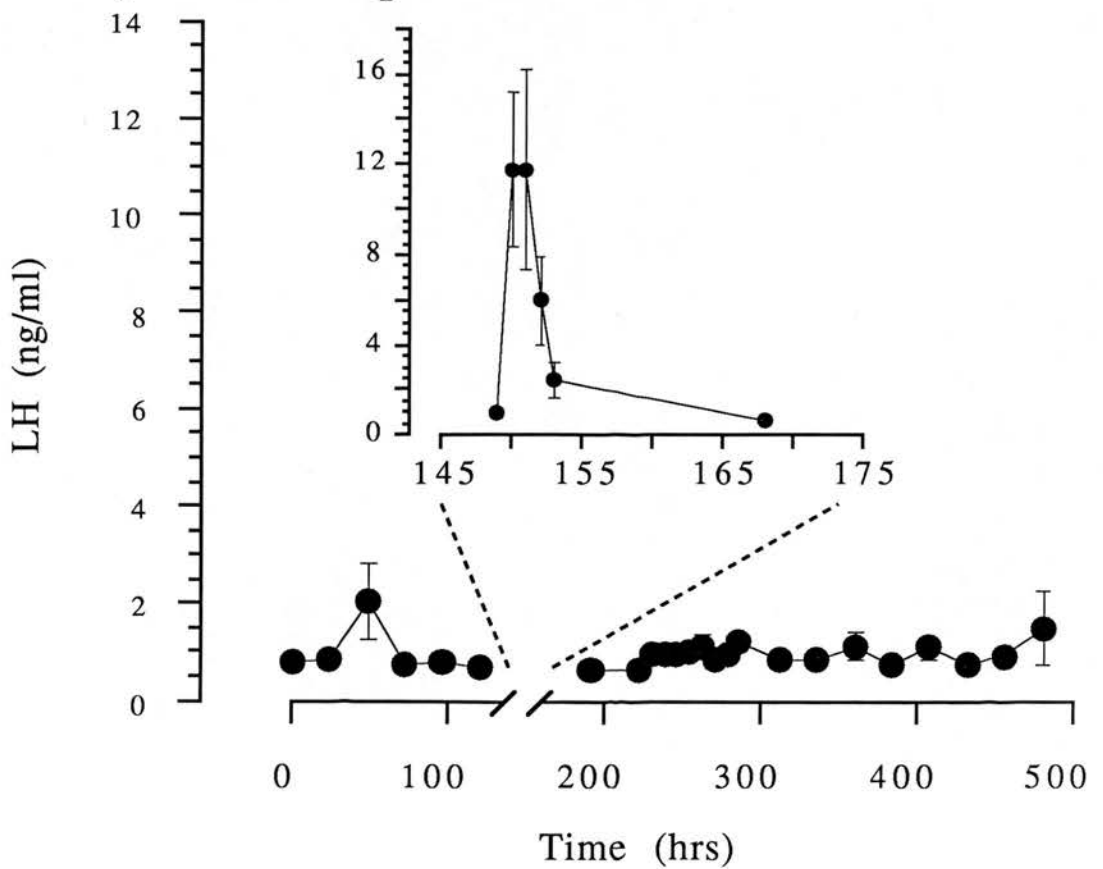
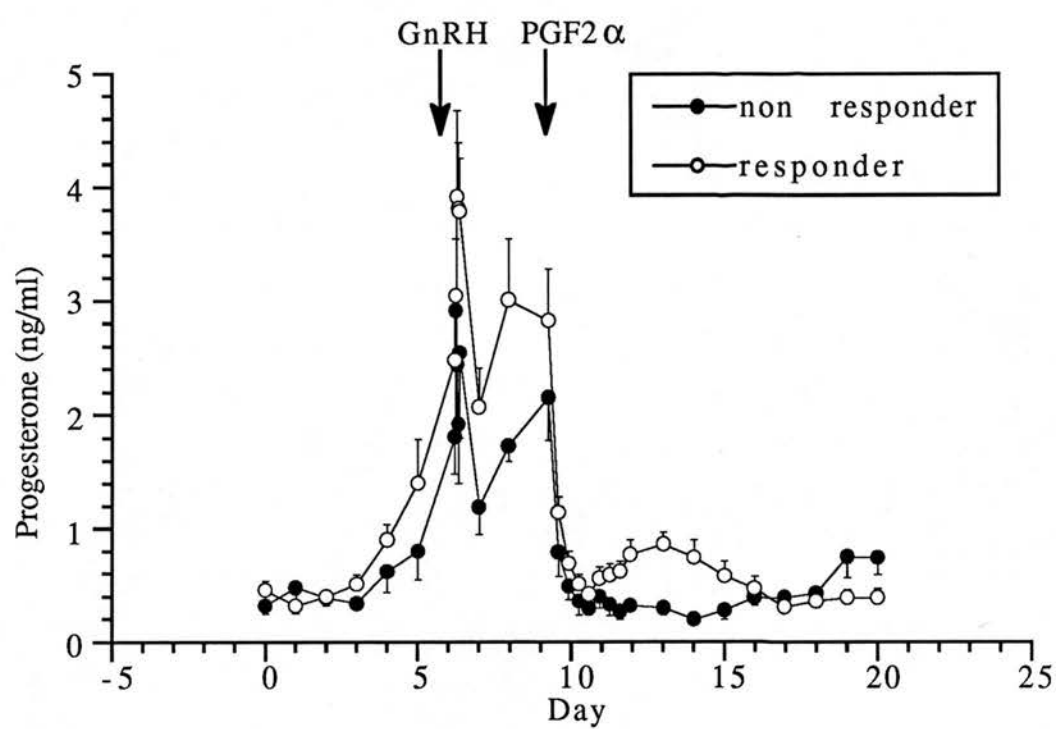


Figure 3.3. Mean (\pm sem) serum LH profiles for the GnRH responders (n=14: upper panel) and non-responders (n=5: lower panel). Time 0 = the time of the first blood sample on the day of the synchronized oestrus following PG2. GnRH was given at 149 hours (approximately 6 days) and PG3 was given at 222 hours (approximately 9 days) after oestrus. The inset graph on each panel shows an expanded portion of the LH profile when the heifers were bled more frequently following GnRH.

Figure 3.4. Mean (\pm sem) serum progesterone profiles for the non-responders (n=5) and GnRH responders (n=14). Day 0 = the day of synchronized oestrus. GnRH was given on Day 6 and PGF_{2 α} (PG3) was given on Day 9.



mean serum progesterone profile prior to GnRH for the non-responders was lower than that of the responders. However, in a subsequent experiment (see Chapter 4; section 4.3.1.5.) progesterone values were similar. Serum LH profiles of responding and non-responding heifers were also not significantly different, and neither was the mean size of the largest follicle on the day of GnRH administration. However in Great Britain (Savio *et al.*, 1988; Gong, Bramley & Webb, 1991) and the U.S.A. (Sirois & Fortune, 1988) 80% of cattle appeared to exhibit 3 waves of follicular growth and development, each wave being characterised by the emergence of a large, dominant follicle. The remaining 20% exhibited usually 2, or exceptionally 4, waves of follicular growth. In animals with 2-wave cycles it is possible that the first dominant follicle will take longer to achieve ovulatory competence. However no significant differences were detected in the day of maximum diameter or duration of growth (Savio *et al.*, 1988), nor in the lengths of the growing and static phases (Ginther *et al.*, 1989a) of the first dominant follicle, in heifers with 2 or 3 follicular waves. As a result of the experimental design we were unable to test the hypothesis that the non-responders were destined to have only 2 follicular waves in the oestrous cycle.

In the present study the induction of an additional CL was associated with a delayed return to oestrus in the GnRH responders following PG3. In a study by Sirois & Fortune (1988) in which animals were monitored by real-time ultrasonography, the relative size of the preovulatory follicle at the completion of luteolysis was negatively correlated with the interval between the end of luteolysis and the LH surge, suggesting that the length of the follicular phase is determined by the size of the largest follicle at the beginning of the follicular phase. However in the present study the delay cannot be considered wholly attributable to the lack of a large, oestrogen

secreting, dominant follicle on the ovary following PG3, because of the significant persistence of luteal function. Interestingly, a similar delay (3.4 days) in return to oestrus following PGF_{2α} has been observed in cows pretreated with a GnRH agonist compared with saline-treated controls (Macmillan, Day, Taufa, Peterson & Pearce, 1985b). However in a similar experiment when ovariectomy was performed 24 hours after the GnRH agonist no secondary CL were observed. These results are not in agreement with our observations in which animals pretreated with GnRH, but without an induced CL, had no delay in return to oestrus following PGF_{2α}; oestrus occurring as expected between 3 and 5 days later (Dailey, James, Inskeep & Washburn, 1983). Thus our results do not support the hypothesis that GnRH-induced LH release has a protective effect on the endogenous mid-cycle CL.

The GnRH-induced CL did not appear to be sensitive to the luteolytic effect of PGF_{2α} on Day 3 of the induced cycle, although its lifespan was limited when compared to the spontaneously-formed CL of the natural cycle. PGF_{2α} is luteolytic when injected i-m or infused into the uterine horn ipsilateral to the CL from 5 days after oestrus in the cow (Inskeep, 1973). Regardless of the route of administration, a single treatment with PGF_{2α} was unable to initiate regression of the bovine CL during the first 4 days of the oestrous cycle. The mechanism whereby the newly-formed CL acquires sensitivity to PGF_{2α} may be related to the induction of PGF_{2α} receptors on the luteal cell membrane. As bovine luteal PGF_{2α} receptors increase progressively from Day 3 to a maximum on Day 20 (Rao *et al.*, 1979) the CL becomes increasingly susceptible to the lytic action of PGF_{2α} (Wakeling & Green, 1981). However hCG can override the luteolytic signal of PGF_{2α} and prevent PGF_{2α}-induced regression in sheep (Bolt, 1979).

It is possible that PGF_{2α} given in the induced cycle 3 days after GnRH, while not causing immediate regression of the induced CL, may partially

compromise its function. Howard and Britt (1990) observed that CL induced in 13 of 16 heifers by treatment with hCG on Day 10 of the oestrous cycle regressed after exogenous $\text{PGF}_{2\alpha}$ given 2 days after ovulation. They suggested that hCG increased the ratio of large ($\text{PGF}_{2\alpha}$ -responsive) luteal cells to small luteal cells in the induced CL. Alternatively, the reduced magnitude of the exogenous GnRH-induced LH surge (50-60% of the spontaneous surge: Webb, 1977; Zolman, Convey, Britt & Hafs, 1973) may have contributed to the premature demise of the induced CL, by reducing luteal LH receptor occupancy and hence advancing induction of $\text{PGF}_{2\alpha}$ receptors. Furthermore, CL formation in a progestational environment may influence subsequent luteal lifespan. When spontaneously-formed CL were exposed to a progestational environment during formation, oestrous cycles and luteal lifespan were shortened (Garrett, Geisert, Zavy, Gries, Wetteman & Buchanan, 1988; Battista, Rexroad & Williams, 1984). However, such CL were not more responsive to $\text{PGF}_{2\alpha}$ (Battista *et al.*, 1984).

Finally, the presence of a large follicle on the ovary may influence the lifespan of the induced CL. The dominant follicle is an important source of oestrogen (Staigmiller *et al.*, 1982; Ireland, Fogwell, Oxender, Ames & Cowley, 1984; Fortune, Sirois & Quirk, 1988), with the concentration of oestradiol-17 β in follicular fluid increasing with follicular size (Ireland, Coulson & Murphree, 1979). Following the demise of the spontaneously-formed CL, oestrogen output by the next wave of growing follicles is low, pituitary gonadotrophin output is increased and the induced CL is maintained. However it is suggested that when one of the pool of growing follicles emerges as the dominant follicle and oestrogen output increases, progesterone secretion by the induced CL may be inadequate to inhibit the positive feedback of oestradiol from the dominant follicle acting at the level of the hypothalamus/pituitary gland. Consistent with this hypothesis is the

observation that the delay from GnRH treatment to oestrus following PG3 in the GnRH responders corresponds approximately to the time for a new wave of follicular development to occur (Ginther *et al.*, 1989a).

The progesterone profiles for the GnRH responders indicated that the induced CL was indeed functional, although progesterone production by the induced CL in this study was relatively low compared with that of the spontaneously-formed CL (Figure 3.4.). It is possible that PG3 may influence progesterone secretion by the induced CL. However, this appears unlikely since mean progesterone concentrations and onset of subsequent oestrus were similar in control and treated heifers, despite progesterone secretion during the first week of the cycle being lower in heifers given 25 mg PGF_{2α} on Day 3 with repeated doses 12 and 24 hours later, than in saline-treated animals (Helmer & Britt, 1987). Furthermore, in a subsequent experiment (see Chapter 5) mean total progesterone production in the four days following lysis of the endogenous CL with PG3, was similar for animals with an induced CL to animals with a spontaneously-formed CL of the same age.

In summary, exogenous GnRH administered to heifers in the early luteal phase induced an additional ovulation in approximately 75% animals. Following PGF_{2α} in the mid-luteal phase, the spontaneously-formed CL underwent luteolysis while the induced CL did not, leading to a delay in return to oestrus associated with a persistence of luteal function. In conclusion these results have shown that the CL induced by the administration of GnRH in the early luteal phase does not control its own lifespan, but that its demise may be associated with an endogenous signal from the dominant follicle. The reason for the premature demise of the induced CL warrants further investigation.

CHAPTER 4

MANIPULATION OF THE LIFESPAN OF THE GnRH-INDUCED CORPUS LUTEUM IN HEIFERS WITH BOVINE FOLLICULAR FLUID

4.1.Introduction

Luteolysis of the CL, with subsequent behavioural oestrus, is a well recognised phenomenon in the non-pregnant heifer with normal oestrous cycles. Whereas the hormone profiles around luteolysis (Peters, 1985a,b) and the histological changes in the CL (Rajakowski, 1960) are well documented, the mechanism(s) which controls the lifespan of the CL and the factor(s) which are responsible for its demise are not fully understood. There is good evidence that $\text{PGF}_{2\alpha}$ of uterine origin is the endogenous agent responsible for triggering luteal regression in cattle (Knickerbocker *et al.*, 1988). However, a number of observations are difficult to reconcile with the above concept. The bovine CL produces relatively large amounts of prostanoids including $\text{PGF}_{2\alpha}$, raising the question as to why the CL needs to rely on $\text{PGF}_{2\alpha}$ of uterine origin for its regression (Hansel & Dowd, 1986). Administration of progesterone on Days 2-5 of the oestrous cycle, at doses which gave a plasma progesterone profile comparable to that on Days 5-9 of a control cycle, shortened the inter-oestrous interval by 5 days (Garrett *et al.*, 1988). The decline in progesterone in the treated animals was associated

with an advanced release of $\text{PGF}_{2\alpha}$. However, the relationship between the first decrease in progesterone and the initial pulse release of $\text{PGF}_{2\alpha}$ (measured as PGFM, the primary metabolite of $\text{PGF}_{2\alpha}$) was not always well correlated. Although providing evidence for the involvement of $\text{PGF}_{2\alpha}$ in luteolysis, these results possibly implicate another factor as the trigger of the process (Garrett *et al.*, 1988).

The close spatial relationship between follicular and luteal elements in the ovary, the waves of follicular growth and development, and the emergence of a large (or dominant) follicle with ovulatory competence around the time of luteolysis, provide circumstantial evidence for a role of follicles and/or follicular products, particularly oestradiol, in luteal regression. This effect could be mediated either by a direct action on the CL, or indirectly, by changing the secretion of prostaglandins and/or gonadotrophins. Destruction of ovarian follicles by electrocautery, followed by X-irradiation of the ovaries during dioestrus, prolongs the lifespan of CL in heifers (Villa-Godoy *et al.*, 1985; Fogwell *et al.*, 1985; Hughes *et al.*, 1987). Furthermore, exogenous $\text{PGF}_{2\alpha}$ failed to induce rapid luteal regression in X-irradiated animals where follicular growth had been inhibited (Hughes *et al.*, 1987). In cycling heifers, oestrogen treatment has also been shown to reduce the length of the oestrous cycle (Wiltbank, 1966). Complete luteal regression could be induced in intact heifers with a dose of oestradiol that produced only a small decrease in weight and total progesterone content in the CL of hysterectomized animals (Brunner *et al.*, 1969), suggesting that the luteolytic action of oestradiol may require the presence of the uterus. Furthermore, the luteolytic response following oestradiol injection was associated with spontaneous spikes of PGFM, that were similar in their timing to those seen in control heifers undergoing luteolysis (Thatcher *et al.*, 1986). In addition to the effects on uterine $\text{PGF}_{2\alpha}$ secretion, oestrogen

may interact with luteal $\text{PGF}_{2\alpha}$ independently of the uterus to reduce luteal function (Gengenbach *et al.*, 1977). Complete luteolysis has been achieved following treatment of hysterectomized ewes with 3.5 mg $\text{PGF}_{2\alpha}$ and 750 μg oestradiol benzoate, whereas $\text{PGF}_{2\alpha}$ given alone was not luteolytic, and oestradiol benzoate given alone had only marginal effects on luteal function.

As a result of the observations made in Chapter 3, we were interested to investigate the reasons for the premature demise of the induced CL. Possible factors controlling the lifespan of the induced CL include: the presence of a large dominant follicle; oestrogen production by the dominant follicle; non-steroidal factor(s) produced by the dominant follicle; exogenous prostaglandin $\text{F}_{2\alpha}$ compromising luteal function; or a combination of these factors. Three experiments were carried out to examine the role of the dominant follicle in the demise of the induced CL, using the same experimental model as described in Chapter 3 (see section 3.2.2.). Bovine follicular fluid was used to suppress the growth of the dominant follicle, and the function of the induced CL was monitored. In Experiment 1, the treatment period was 3 days in length and commenced on Day 7 after GnRH. To investigate the relationships between time of onset and duration of bFF treatment, and effects on luteal function, the treatment period was increased to 4 or 8 days in Experiment 2 and commenced on Day 5 after GnRH. Following results obtained from this experiment, the 8-day treatment period was repeated in Experiment 3 with an increased number of animals per group.

4.2. Materials and Methods

4.2.1. Experiment 1

4.2.1.1. Animals

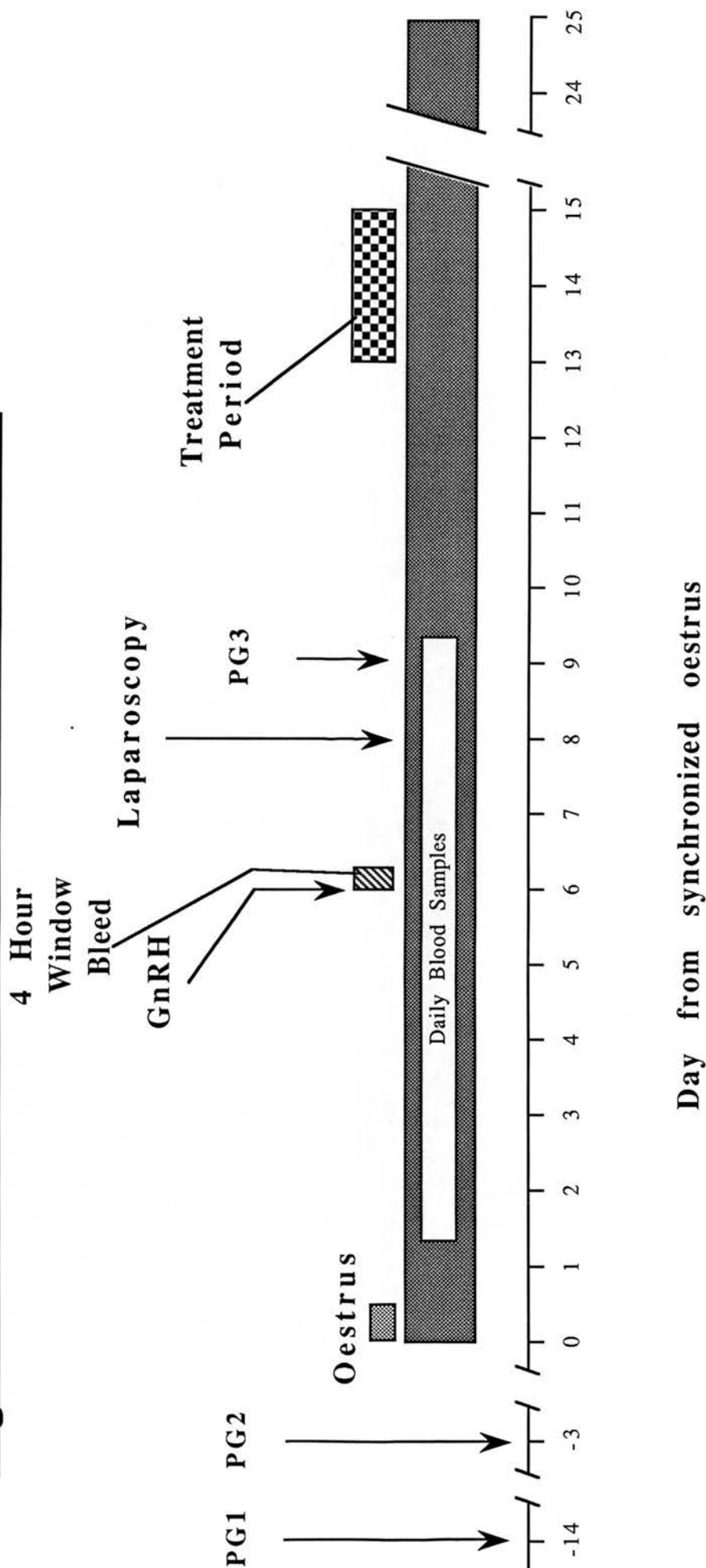
Mature Hereford cross Friesian heifers were housed in straw yards on the Institute Large Animal Unit and fed a maintenance ration of hay and grass nuts.

4.2.1.2. Experimental procedure

The experimental protocol is shown in Figure 4.1. and was initially similar to the procedure described in Chapter 3 (see section 3.2.2.). Briefly the oestrous cycles of 17 heifers were synchronized with 2 injections of 500 µg cloprostenol (Estrumate: Coopers Animal Health, Crewe Hall, Cheshire), 11 days apart (PG1 and PG2). Oestrous behaviour following PG2 was monitored by 3 times-daily observation, together with the use of heat detection devices (Kamar Heatmount Detectors: Kamar Inc., Steamboat Springs, CO, USA). Oestrus was confirmed retrospectively by daily blood sampling for serum progesterone concentrations. Six days after the synchronized oestrus (oestrus = Day 0) all heifers received 0.5 mg gonadorelin (GnRH; Fertagyl: Intervet Laboratories Ltd., Science Park, Milton Rd., Cambridge) by i.m. injection, which was followed by a 4-hour window bleed to characterise the LH response.

In preparation for laparoscopy, food was withheld for 48 hours and water was withheld for 24 hours. All heifers were subjected to sublumbar laparoscopy 2 days after the gonadorelin injection. Animals with no new ovulation point were classified as non-responders. Heifers with a fresh

Figure 4.1. PROTOCOL FOR EXPERIMENT 1



ovulation point, and hence a new induced CL, were classified as GnRH responders (subsequent cycle referred to as the induced cycle, where day of GnRH injection = Day 0). Twenty-four hours after laparoscopy and 3 days after GnRH administration, all heifers received 500 µg cloprostenol (PG3) to remove the spontaneously-formed CL. Thirteen heifers with induced CL (responders) were randomly divided into 2 groups between Days 7-9 (the treatment period) after GnRH: Group 1 (n=7) received 10 ml 0.9% saline i.v. 3 x daily for 3 days; Group 2 (n=6) received 10 ml bFF i.v. 3 x daily for 3 days. The non-responders (n=4) received 10 ml 0.9% saline i.v. 3 x daily for the treatment period. Oestrous behaviour was monitored as before; blood sampling frequency was increased to 3 times-daily for 10 days following PG3 to characterise the FSH response, and then continued once-daily until all heifers had shown oestrus. Animals were monitored by rectal ultrasonography once-daily preceding GnRH administration to record follicular status, and on Day 6 after GnRH to confirm the presence or absence of the induced CL. In all heifers with an induced CL, the growth of the largest follicle was followed by daily ultrasonography from the start of the treatment period until the subsequent oestrus, to confirm the suppression of the growth of the dominant follicle in the bFF-treated heifers.

4.2.1.3. Hormone radioimmunoassays

Blood samples were collected by jugular venepuncture, and serum harvested and stored at -20°C for subsequent measurement of peripheral LH, FSH and progesterone concentrations.

4.2.1.3.1. LH assay

Samples from this study were assayed for LH in one assay. The sensitivity of the assay was 50.0 pg/tube (0.5 ng/ml) and the intra-assay coefficient of variation was 8.0%.

4.2.1.3.2. FSH assay

The mean sensitivity of the assays was 1.7 ng/tube (6.8 ng/ml). Samples were analysed in 2 assays. The inter- and intra-assay coefficients of variation were 6.5% and 9.6% respectively.

4.2.1.3.3. Progesterone assay

Samples from this study were assayed for progesterone in 3 assays. The mean sensitivity of the assays was 19.7 pg/tube (0.4 ng/ml) and the inter- and intra-assay coefficients of variation were 15.9% and 10.8% respectively.

4.2.2. Experiment 2

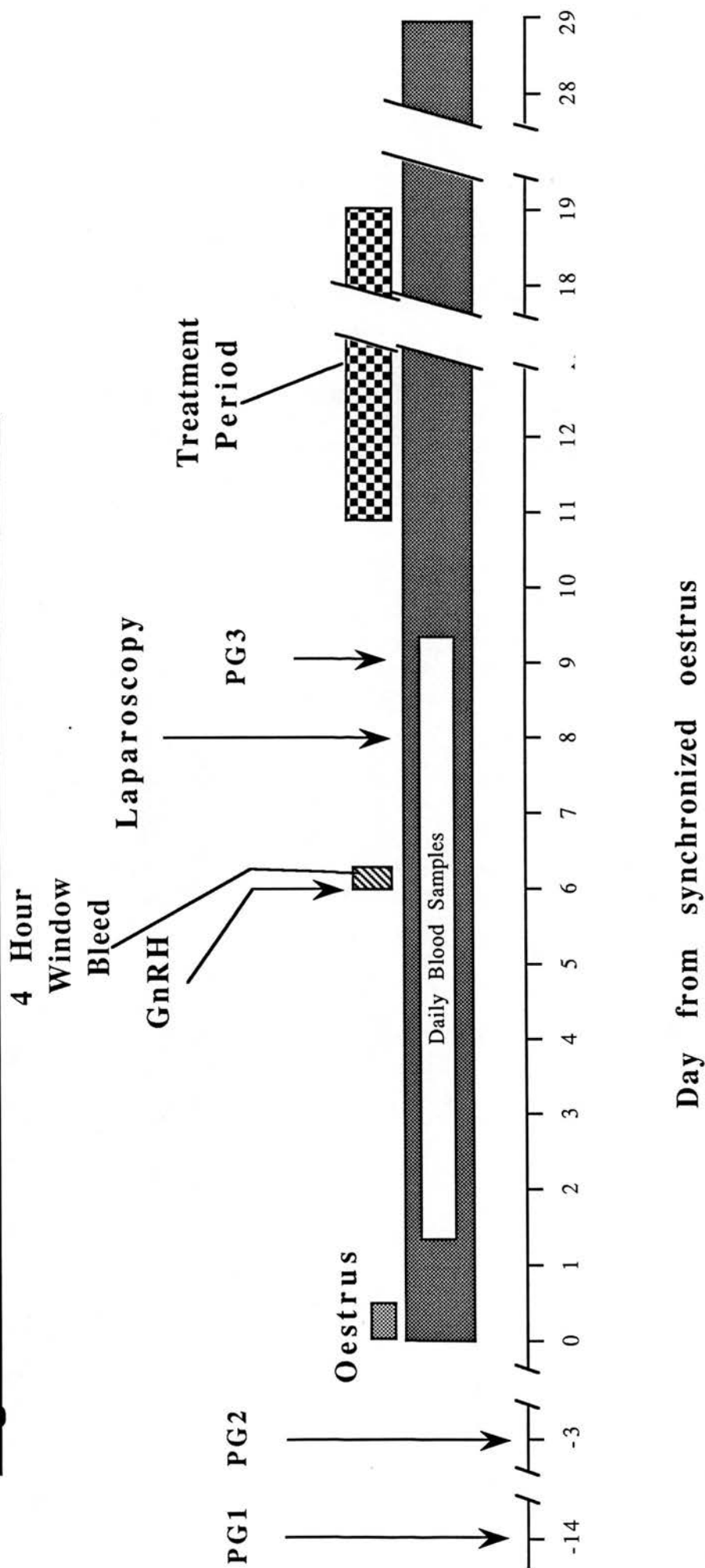
4.2.2.1. Animals

Sexually mature growing heifers were housed in straw yards on the Institute Large Animal Unit and fed a maintenance ration of hay and grass nuts. Heifers were approximately 15-18 months old and of mixed breed (principally Angus cross, Simmentel cross, Blonde d'Aquitaine cross, Limousin cross and Charolais cross).

4.2.2.2. Experimental procedure

The experimental protocol is shown in Figure 4.2. and was initially similar to the procedure described in section 4.2.1.2. of this chapter. Briefly the oestrous cycles of 20 heifers were synchronized with 2 injections of 500 μ g cloprostenol (PG1 and PG2) and on Day 6 (synchronized oestrus = Day 0) all heifers received 0.5 mg gonadorelin followed by a 4-hour window bleed to monitor the rise in serum LH concentrations. The GnRH challenge was followed 48 hours later by laparoscopy to determine the ovulatory response. Three days after GnRH all heifers received 500 μ g cloprostenol (PG3) to remove the spontaneously-formed CL. Eighteen heifers with an induced CL were split into 3 groups between Days 5-12 (the treatment period) after GnRH: Group 1 (n=6) received 10 ml 0.9% saline i.v. 3 x daily for 8 days; Group 2a (n=6) received 10 ml bFF i.v. 3 x daily for 4 days followed by 10 ml 0.9% saline i.v. 3 x daily for 4 days; Group 2b (n=6) received 10 ml bFF i.v. 3 x daily for 8 days. The non-responders (n=2) received 10 ml 0.9% saline i.v. 3 x daily for the treatment period. Oestrous behaviour was monitored as before; blood sampling frequency was increased to 3 times-daily for 10 days from PG3 to characterise the FSH response, and then continued once-daily until all heifers had shown oestrus. All animals were monitored by rectal ultrasonography once-daily for 5 days preceding GnRH administration to record follicular status, and on Day 6 after GnRH to confirm the presence or absence of the induced CL. Growth of the largest follicle was followed by daily ultrasonography in 6 heifers with an induced CL (2 from each of Groups 1, 2a and 2b) from the start of the treatment period until the subsequent oestrus, to confirm the suppression of the growth of the dominant follicle in the bFF-treated heifers.

Figure 4.2. PROTOCOL FOR EXPERIMENT 2



4.2.2.3. Hormone radioimmunoassays

Blood samples were collected by jugular venepuncture, and serum harvested and stored at -20°C for subsequent measurement of peripheral LH, FSH, progesterone and oestradiol concentrations.

4.2.2.3.1. LH assay

Samples from this study were assayed for LH in 2 assays. The mean sensitivity of the assays was 56.5 pg/tube (0.6 ng/ml) and the inter- and intra-assay coefficients of variation were 11.9% and 14.2% respectively.

4.2.2.3.2. FSH assay

Samples from this study were assayed for FSH in 2 assays. The sensitivity of the assays was 1.6 ng/tube (6.3 ng/ml) and the inter- and intra-assay coefficients of variation were 3.4% and 7.8% respectively.

4.2.2.3.3. Progesterone assay

Samples from this study were assayed for progesterone in 3 assays. The mean sensitivity of the assays was 24.9 pg/tube (0.5 ng/ml) and the inter- and intra-assay coefficients of variation were 13.8% and 17.0% respectively.

4.2.2.3.4. Oestradiol assay

Samples from this study were assayed for oestradiol in 6 assays with a mean extraction efficiency of $53 \pm 2\%$. The mean sensitivity of the assays was 0.9 pg/tube and the inter- and intra-assay coefficients of variation were 7.7% and 9.2% respectively.

4.2.3. Experiment 3

4.2.3.1. Animals

Mature Hereford cross Friesian heifers were housed in straw yards on the Institute Large Animal Unit and maintained under the same conditions as Experiments 1 and 2.

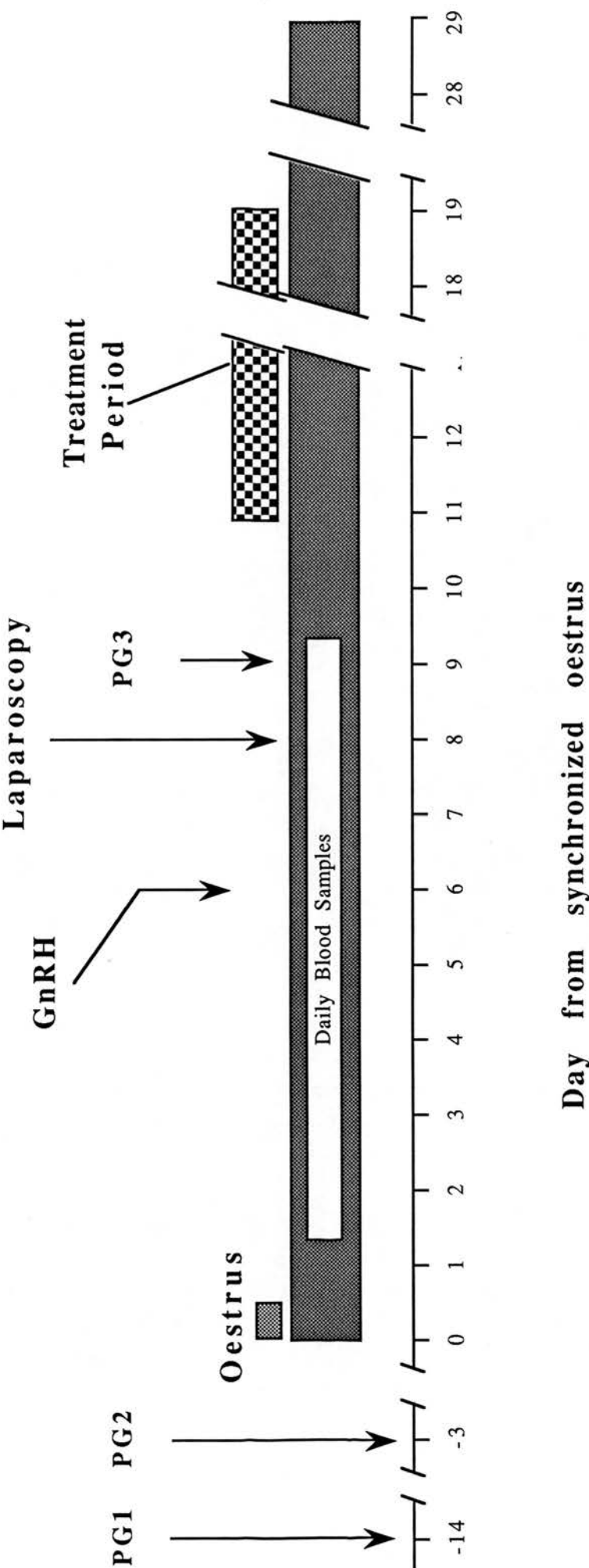
4.2.3.2. Experimental procedure

Twenty-two heifers were subject to the experimental protocol shown in Figure 4.3. which was initially similar to the procedure described in section 4.2.1.2. of this chapter, omitting the 4-hour window bleed as the LH response has been previously characterized (see section 3.3.3., Chapter 3, and sections 4.3.1.2. and 4.3.2.2., Chapter 4). Twenty heifers with an induced CL were split into 2 groups between Days 5-12 (the treatment period) after GnRH: Group 1 (n=11) received 10 ml 0.9% saline i.v. 3 x daily for 8 days; Group 2 (n=9) received 10 ml bFF i.v. 3 x daily for 8 days. The non-responders (n=2) received 10 ml 0.9% saline i.v. 3 x daily for the treatment period. Animals were monitored as before until the onset of the subsequent oestrus. Rectal ultrasonography was used to confirm the presence of the induced CL 6 days after the GnRH challenge.

4.2.3.3. Hormone radioimmunoassays

Blood samples were collected by jugular venepuncture, and serum harvested and stored at -20°C for subsequent measurement of peripheral progesterone and oestradiol concentrations.

Figure 4.3. PROTOCOL FOR EXPERIMENT 3



4.2.3.3.1. Progesterone assay

Samples from this study were assayed for progesterone in 3 assays. The mean sensitivity of the assays was 7.5 pg/tube (0.2 ng/ml) and the inter- and intra-assay coefficients of variation were 13.4% and 9.0% respectively.

4.2.3.3.2. Oestradiol assay

Samples from this study were assayed for oestradiol in 6 assays with a mean extraction efficiency of $55 \pm 1\%$. The mean sensitivity of the assays was 0.8 pg/tube and the inter- and intra-assay coefficients of variation were 14.8% and 12.5% respectively.

4.2.4. bFF preparation

bFF was prepared as described in Chapter 2 (see section 2.7.1.). Charcoal treatment reduced concentrations of oestradiol and progesterone in the follicular fluid from 305.3 to 7.6 ng/ml and from 628.9 to 15.2 ng/ml, respectively.

4.2.5. Statistical analysis

All data are presented as means \pm sem. Differences between the treatment groups in the interval from PG3 to oestrus were tested by Student's *t* test.

in Experiments 1 and 2
LH profiles were analyzed following GnRH during the LH surge, and total amount of hormone secreted was determined from the area under the peak. Mean LH concentrations for the bFF-treated responders were determined in the treatment period (Experiment 1: Days 7-9 after GnRH; Experiment 2: Days 5-8 and 5-12 after GnRH), and compared to the means for

the saline-treated responders in the interval between the onset of the treatment period and the subsequent oestrus.

FSH profiles were analyzed on a daily basis following PG3 when mean serum progesterone first fell below 1 ng/ml, to the sample preceding the mean day of oestrus for each of the groups in Experiments 1 and 2. Differences in total FSH production between saline-treated and bFF-treated responders in Experiment 1, between saline treated and both 4-day and 8-day bFF-treated responders in Experiment 2, and between 4-day and 8-day bFF-treated responders in Experiment 2 were tested using Student's *t* test. For the bFF-responders, mean FSH concentrations during the treatment period were compared to mean FSH concentrations 48-hour post-treatment using Student's *t* test.

Progesterone profiles were analyzed on a daily basis in the period following PG3, from when mean serum progesterone concentration had fallen below 1 ng/ml, to the next nadir when the serum progesterone concentration had fallen below the minimum detectable limit for the assay. Differences in total progesterone production between saline-treated and bFF-treated heifers with a functional CL (as defined below) in Experiment 1, and between saline-treated and both 4-day and 8-day bFF-treated responders in Experiment 2, were tested using Student's *t* test.

Following the PG3-induced decline in serum progesterone concentration to <1 ng/ml, animals were defined as having a functional induced CL if the subsequent progesterone profile exceeded 1 ng/ml before the onset of oestrus. When the progesterone profile did not exceed 1 ng/ml following the demise of the endogenous CL and the onset of the subsequent oestrus, the induced CL was defined as non-functional. Hunter (1991) has defined normal CL function in ewes as plasma progesterone concentrations of >1 ng/ml for at least 8 days, whereas abnormal CL show a transient rise in plasma progesterone to >0.5 ng/ml followed by a rapid decline. Differences between mean period of progesterone production and mean peak progesterone concentration between saline-treated responders with a functional CL and bFF-responders with a functional CL in Experiments 1 and 2 were tested using Student's *t* test.

Oestradiol profiles in Experiments 2 and 3 were analyzed during the treatment period (Days 5-12 after GnRH). The difference in total oestradiol

production between Groups 1 and 2b in Experiment 2, and between Groups 1 and 2 in Experiment 3, was tested using Student's *t* test incorporating Satterthwaite's approximation for means with differing variances. Differences between daily mean serum oestradiol concentrations for the saline-treated and bFF-treated responders in Experiment 3 were tested by fitting quadratic curves to the oestradiol profiles for the 2 groups during Days 13-17 (Days 7-11 after GnRH) and comparing the fitted values.

Profiles for the ratio of oestradiol:progesterone in Experiments 2 and 3 were analyzed in the treatment period (Days 5-12 after GnRH). The difference in the mean ratio between Groups 1 and 2b in Experiment 2, and between Groups 1 and 2 in Experiment 3, was tested using Student's *t* test incorporating Satterthwaite's approximation for means with differing variances.

4.3.Results

4.3.1. Experiment 1

4.3.1.1. Response to GnRH

Thirteen (76%) of the 17 heifers responded to the GnRH injection with an additional ovulation. The mean interval from PG3 to oestrus was extended by 3.9 days ($P<0.05$) in the saline-treated responders (Group 1) compared to the non-responders, and by 9.8 days ($P<0.05$) in the bFF-treated responders (Group 2) compared to the non-responders. Comparing the bFF-treated group to the saline-treated responders (Group 1), there was an extension in the interval from PG3 to oestrus of 5.7 days in Group 2 ($P<0.001$).

4.3.1.2. Ovarian observations

Ultrasound observations confirmed the emergence of a large follicle from the first wave of follicular growth in the oestrous cycle following the

synchronized oestrus. As in Chapter 3 (see section 3.3.1.) there were no apparent differences between responders and non-responders in the characteristics (size and growth rate) of the large follicle before GnRH treatment, similar to previously published observations (Gong *et al.*, 1993). Ultrasonography from 5 days after GnRH confirmed the presence of an induced CL in the GnRH responders, and followed the emergence of a new wave of follicular growth which coincided approximately with the emergence of the second (post-synchronized oestrus) wave of follicles in the saline-treated GnRH responders. In bFF-treated heifers the emergence of this follicular wave did not occur until after the cessation of bFF treatment.

4.3.1.3. Peripheral LH concentrations

Mean serum LH profiles for the non-responders, saline-treated responders (Group 1), and bFF-treated responders (Group 2), are shown in Figure 4.4. All heifers had a surge release of LH following GnRH. There was no difference in the magnitude of the LH surge between non-responders (12.5 ± 3.2 ng/ml) and responders (12.2 ± 1.1 ng/ml), or in the total amount of hormone secreted. Mean serum LH concentration for the bFF-treated responders in the treatment period was not different from the saline-treated responders (0.9 ± 0.1 ng/ml and 1.1 ± 0.2 ng/ml respectively). Furthermore, the mean serum LH concentration for the responders in the treatment period was similar to the mean luteal phase serum LH concentration in the non-responders (0.8 ± 0.1 ng/ml).

4.3.1.4. Peripheral FSH concentrations

Mean serum FSH profiles for the saline-treated responders (Group 1) and bFF-treated responders (Group 2) are shown in Figure 4.5. Following PG3 the mean serum FSH profile for the saline-treated responders was not different from the mean serum FSH profile for the bFF-treated responders.

Figure 4.4. Mean (\pm sem) serum LH profiles during the window bleed following the GnRH challenge, for the non-responders (n=4: upper panel), saline-treated responders (Group 1; n=7: middle panel) and bFF-treated responders (Group 2; n=6: lower panel). Time 0 = the time of the first blood sample on the day of the synchronized oestrus following PG2. GnRH was given at 149 hours (approximately 6 days) after oestrus.

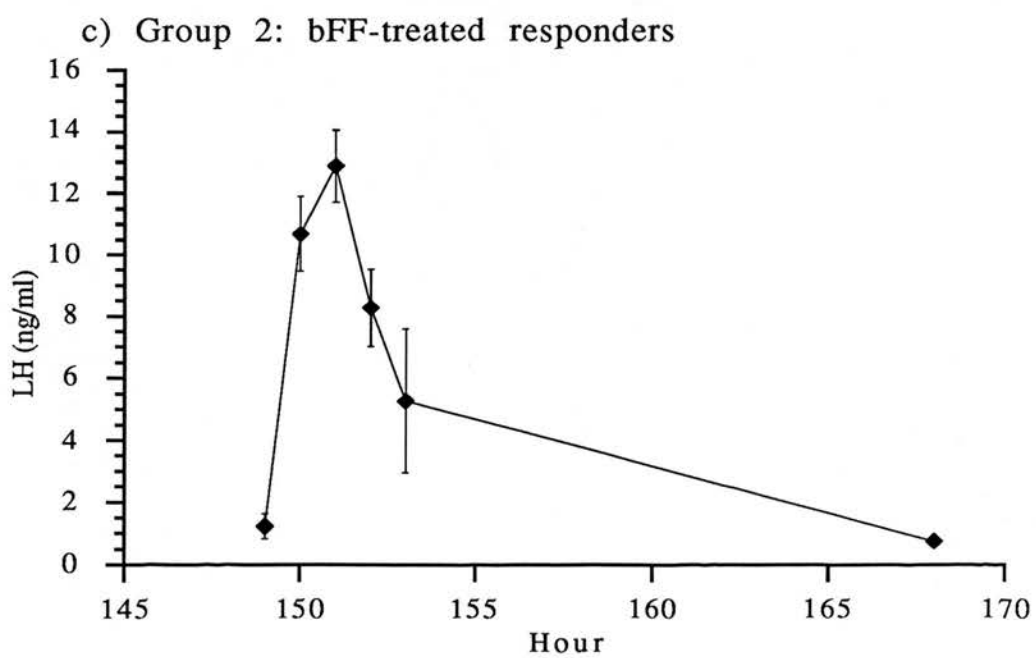
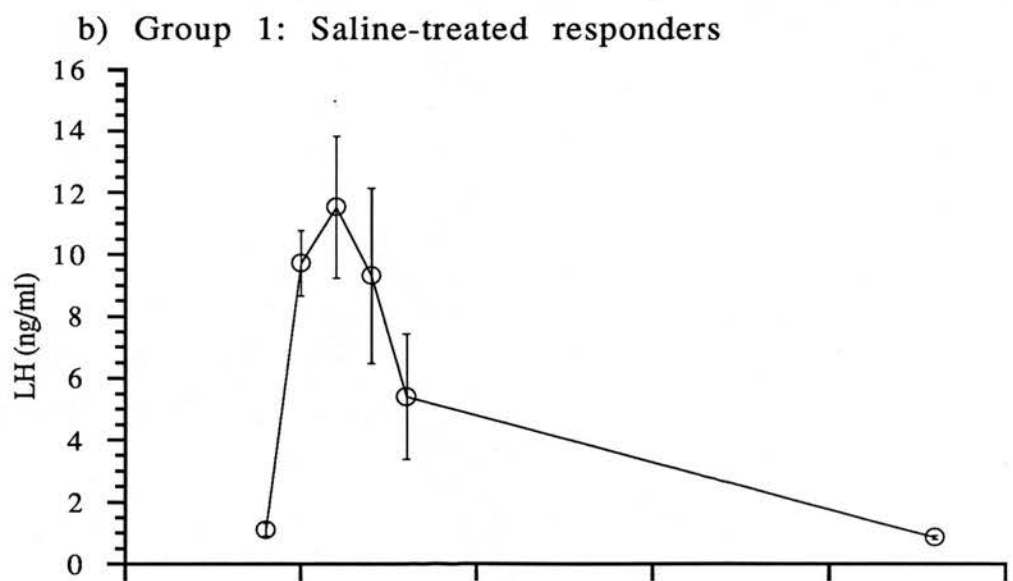
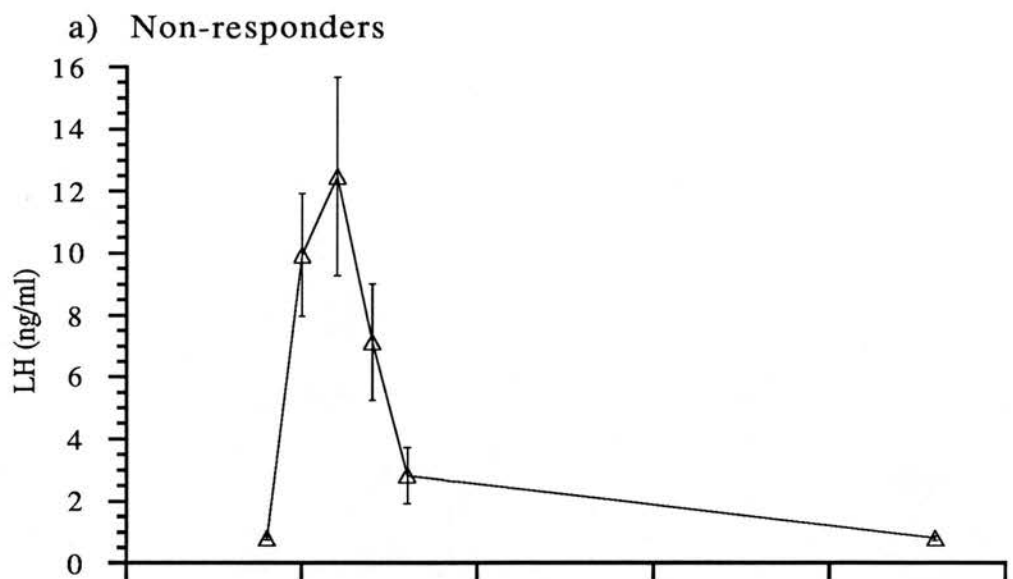
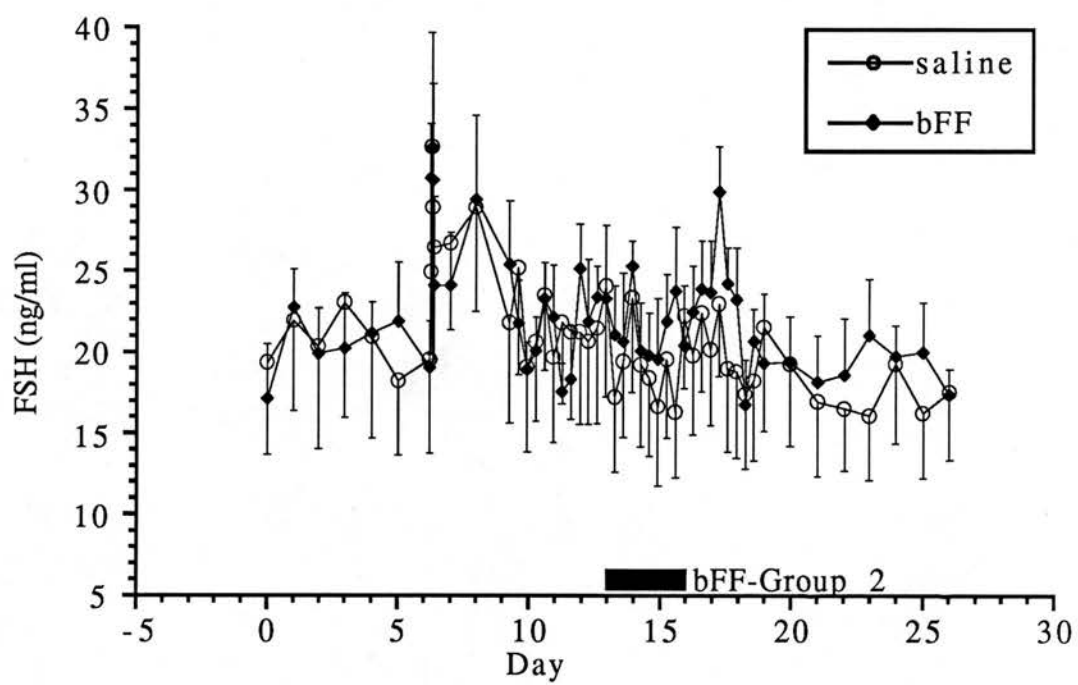


Figure 4.5. Mean (\pm sem) serum FSH profiles for the saline-treated responders (Group 1; n=7) and the bFF-treated responders (Group 2; n=6) in Experiment 1. Day 0 = the day of synchronized oestrus. GnRH was given on Day 6 and PGF₂ α (PG3) was given on Day 9. The bar indicates the period of bFF treatment.



The increase in FSH in bFF-treated responders following cessation of bFF administration was not statistically significant due to between-animal variation in serum FSH concentrations.

4.3.1.5. Peripheral progesterone concentrations

Mean serum progesterone profiles for the non-responders, saline-treated responders (Group 1), and bFF-treated responders (Group 2), are shown in Figure 4.6. Following PG3, mean serum progesterone concentrations fell in all 3 groups and the delay in regression of the induced CL in the saline- and bFF-treated responders was associated with elevated mean progesterone profiles compared to the non-responders. However the differences in total progesterone secreted were not statistically significant due to the small number of non-responders.

Retrospective re-analysis of individual progesterone profiles of the saline- and bFF-treated responders, in which rectal ultrasonography 6 days after GnRH had confirmed the presence of the induced CL, revealed that 6 (55%) of the GnRH responders had a functional CL. Three (60%) of the saline-treated responders (Group 1f) and 3 (50%) of the bFF-treated responders (Group 2f) had a functional induced CL. In Group 2f there was a further delay in regression of the induced CL associated with a significantly increased persistence of luteal function ($P < 0.01$) compared to the mean of the saline-treated responders (Group 1); the other 3 bFF-treated heifers were not different from the saline-treated group (Figure 4.7.). The mean (\pm sem) period of progesterone production from PG3, as defined in section 4.2.5., was 10.0 ± 1.0 days in Group 2f, and extended by approximately 4.5 days when compared to Group 1f (5.4 ± 0.6 days; $P < 0.05$). Mean (\pm sem) peak progesterone concentration in the interval from PG3 to oestrus in Group 2f (2.7 ± 0.1

Figure 4.6. Mean (\pm sem) serum progesterone profiles for the non-responders (n=4: upper panel), saline-treated responders (Group 1; n=7: middle panel) and bFF-treated responders (Group 2; n=6: lower panel) in Experiment 1. Day 0 = the day of synchronized oestrus. GnRH was given on Day 6 and PGF₂ α (PG3) was given on Day 9. Bars indicate the period of bFF or saline treatment.

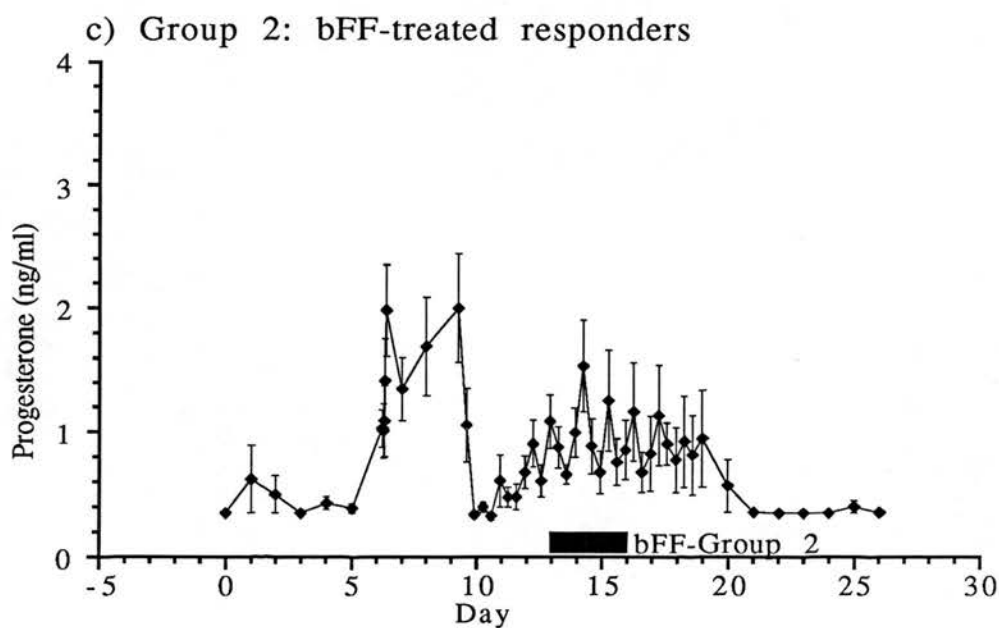
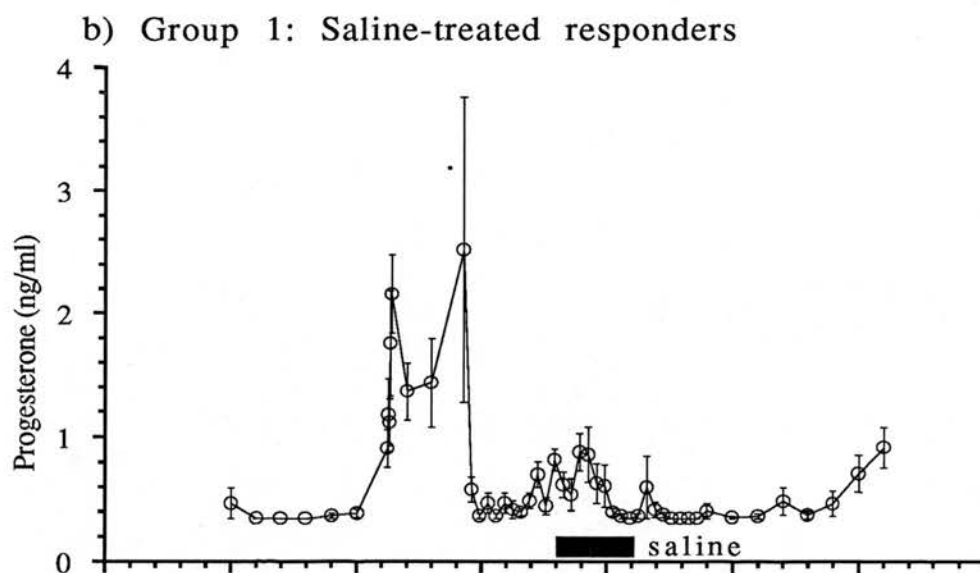
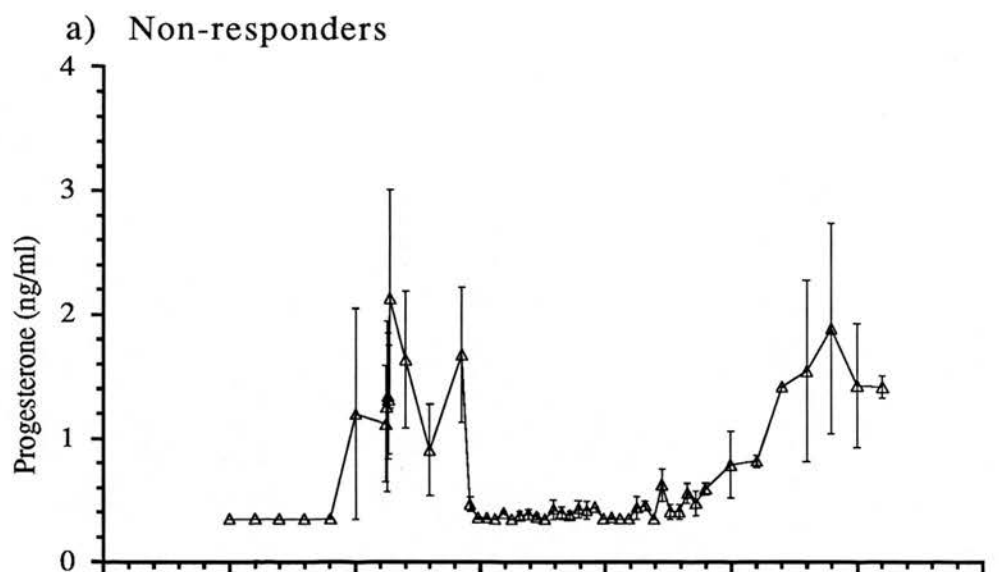
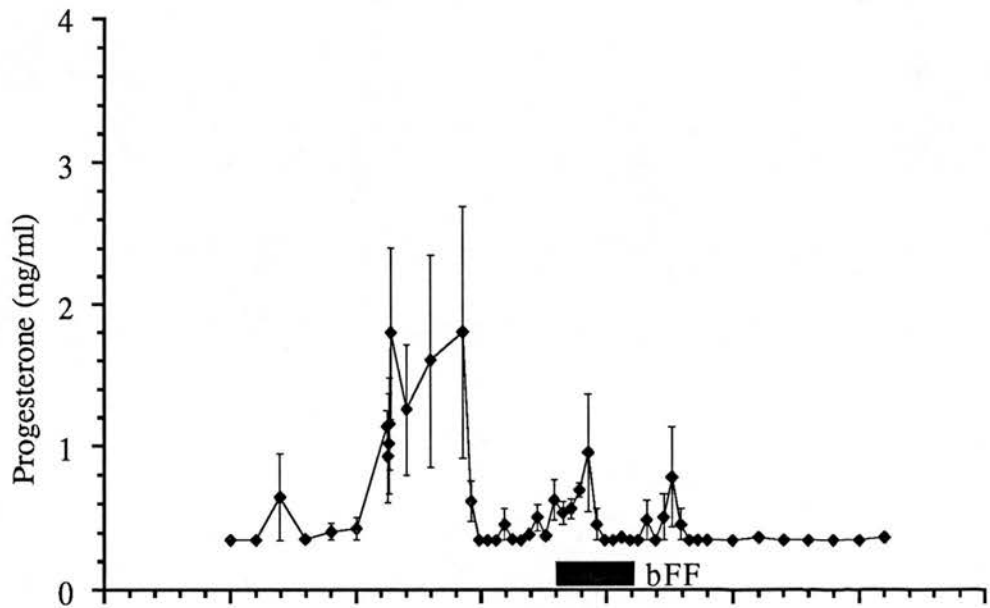
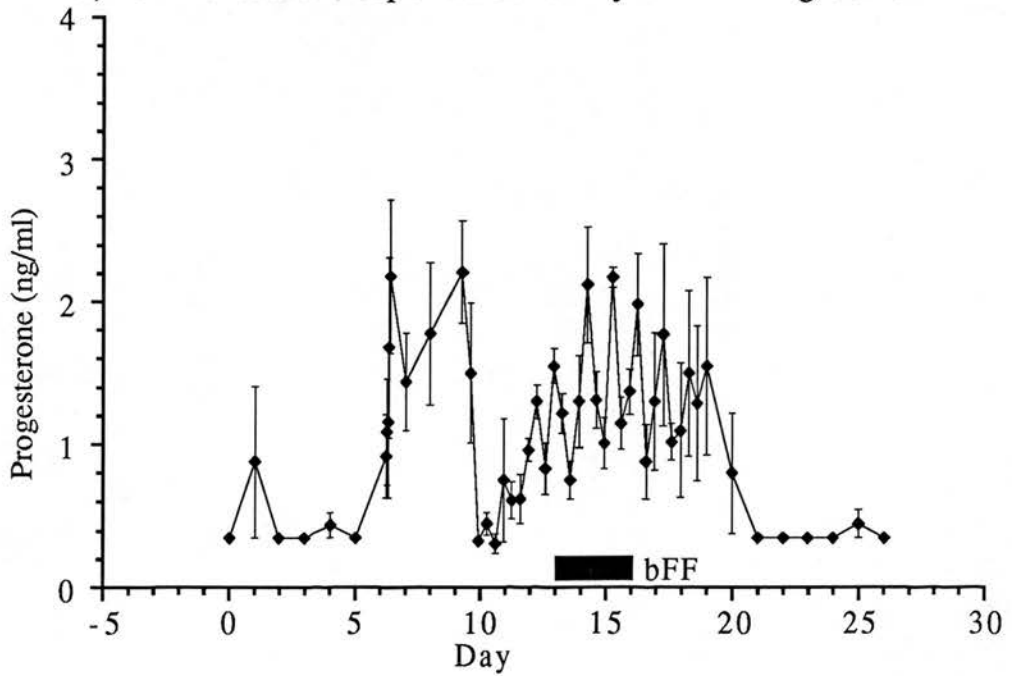


Figure 4.7. Mean (\pm sem) serum progesterone profiles for the bFF-treated responders (n=3: upper panel) with no delay in regression of the induced CL when compared to the saline-treated responders (data not shown), and the bFF-treated responders (n=3: lower panel) with delayed regression of the induced CL when compared to the saline-treated responders (data not shown) in Experiment 1. Day 0 = the day of synchronized oestrus. GnRH was given on Day 6 and PGF₂ α (PG3) was given on Day 9. Bars indicate the period of bFF treatment.

a) bFF-treated responders: no delay in CL regression



b) bFF-treated responders: delayed CL regression



ng/ml) was increased significantly ($P < 0.001$) by approximately 1.5 ng/ml when compared to Group 1f (1.3 ± 0.1 ng/ml).

4.3.2. Experiment 2

Following the results of Experiment 1, Experiment 2 was carried out to investigate the relationships between time of onset and duration of bFF treatment.

4.3.2.1. Response to GnRH

Eighteen (90%) of the 20 heifers responded to the GnRH injection with an additional ovulation. The mean interval from PG3 to oestrus was extended by 3.1 days (non-significant) in the saline-treated responders (Group 1) compared to the non-responders; by 7.5 days ($P < 0.01$) in Group 2a (4-day bFF-treated responders) compared to the non-responders; and by 11.0 days ($P < 0.001$) in Group 2b (8-day bFF-treated responders) compared to the non-responders. Comparing the bFF-treated groups to the saline-treated responders (Group 1), there was an extension in the interval from PG3 to oestrus of 4.4 days in Group 2a ($P < 0.01$) and of 7.9 days in Group 2b ($P < 0.001$).

4.3.2.2. Ovarian observations

Ultrasound observations were similar to Experiment 1 (see section 4.3.1.2.).

4.3.2.3. Peripheral LH concentrations

As in Experiment 1, the 4-hour window bleed confirmed that all heifers had a surge release of LH following GnRH. There was no significant difference in the magnitude of the LH surge between non-responders (13.2 ± 1.0 ng/ml) and responders (16.4 ± 1.7 ng/ml), or in the total amount of hormone secreted. Mean serum LH concentrations for the 4-day and 8-day

bFF-treated responders in the treatment period were not different from the saline-treated responders (0.9 ± 0.2 ng/ml, 1.1 ± 0.2 ng/ml and 0.8 ± 0.3 ng/ml, respectively). As in Experiment 1, mean serum LH concentrations for the responders in the treatment period were not different from the mean luteal phase serum LH concentration in the non-responders (0.9 ± 0.1 ng/ml).

4.3.2.4. Peripheral FSH concentrations

Mean serum FSH profiles for the saline-treated responders (Group 1), the 4-day bFF-treated responders (Group 2a) and the 8-day bFF-treated responders (Group 2b) are shown in Figure 4.8. Although there is an apparent difference in mean serum FSH concentrations between the saline-treated responders and the bFF-treated responders during the period of bFF administration (upper and middle panels), it is unlikely that this is a true effect of the bFF treatment as there is a similar difference in the FSH profiles pre-GnRH administration. Cessation of bFF administration was associated with a significant increase in peripheral concentrations of FSH in the 4-day bFF-treated responders ($P < 0.01$) and the 8-day bFF-treated responders ($P < 0.01$).

4.3.2.5. Peripheral progesterone concentrations

Analysis of progesterone profiles revealed that mean serum progesterone concentrations fell in all heifers with the regression of the endogenous spontaneously-formed CL following PG3, but the presence of the induced CL, confirmed by rectal ultrasonography 6 days after GnRH, was associated with persistence of luteal function in all 3 groups. Mean serum progesterone profiles for the saline-treated responders compared to the 4-day bFF-treated responders (Groups 1 & 2a: upper panel) and the 8-day bFF-treated responders (Groups 1 & 2b: middle panel) are shown in Figure 4.9. A

Figure 4.8. Mean (\pm sem) serum FSH profiles for the saline-treated responders (n=6) compared to the 4-day bFF-treated responders (n=6) (Groups 1 & 2a: upper panel); the saline-treated responders (n=6) compared to the 8-day bFF-treated responders (n=6) (Groups 1 & 2b: middle panel); and the 4-day bFF-treated responders (n=6) compared to the 8-day bFF-treated responders (n=6) (Groups 2a & 2b: lower panel). Day 0 = the day of synchronized oestrus. GnRH was given on Day 6 and PGF₂ α (PG3) was given on Day 9. Bars indicate the period of bFF treatment.

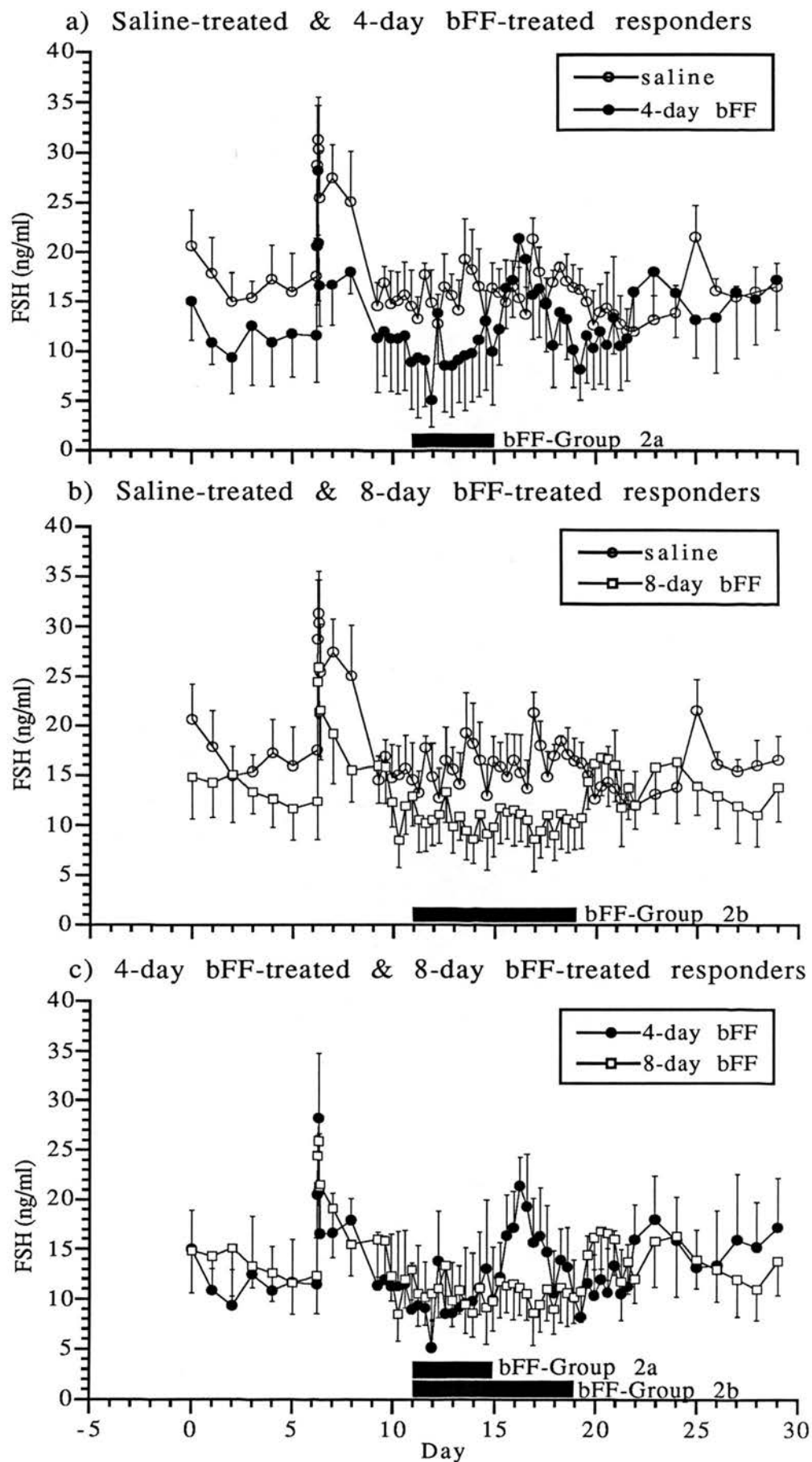
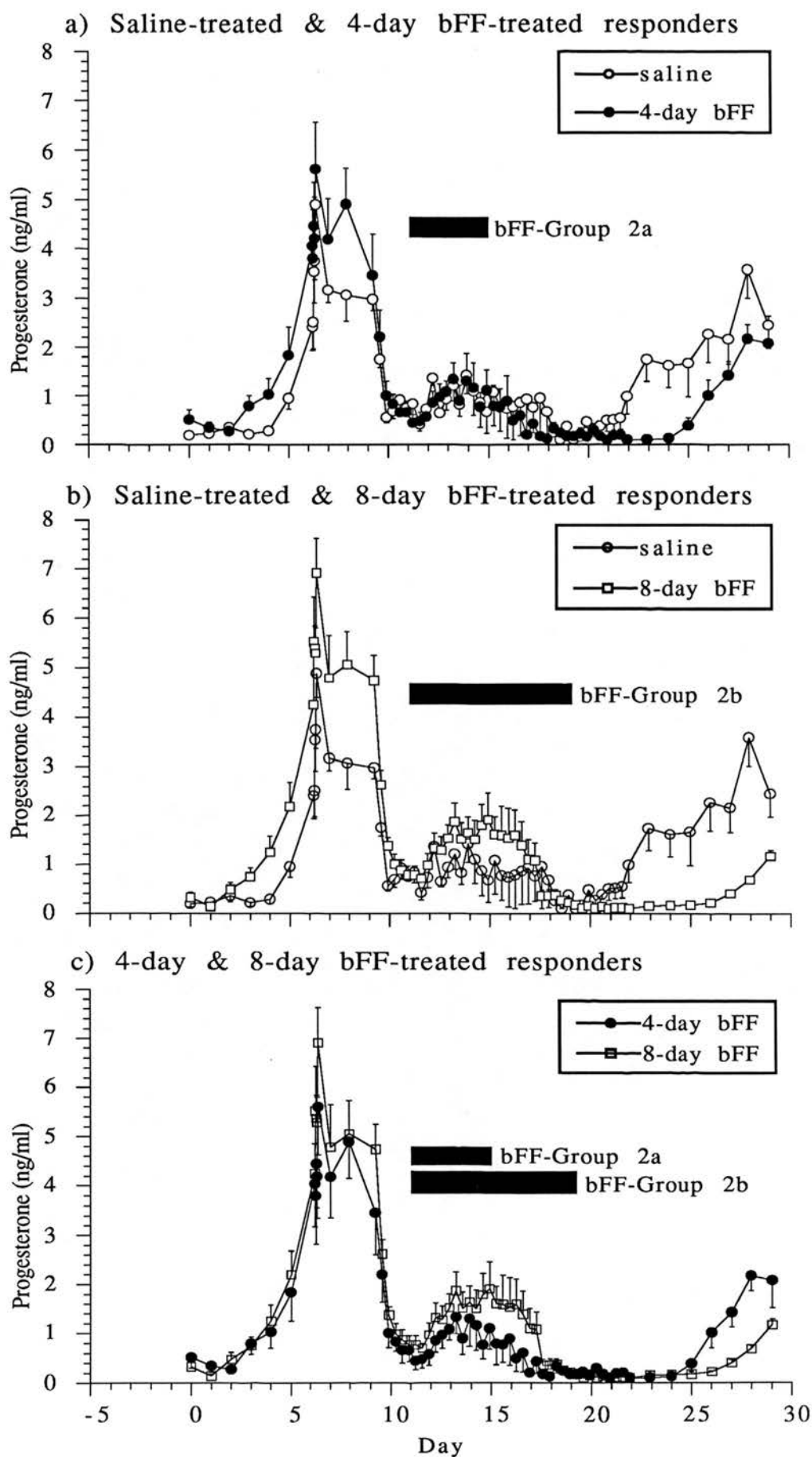


Figure 4.9. Mean (\pm sem) serum progesterone profiles for the saline-treated responders (n=6) and the 4-day bFF-treated responders (n=6) (Groups 1 & 2a: upper panel); the saline-treated responders (n=6) and the 8-day bFF-treated responders (n=6) (Groups 1 & 2b: middle panel); and the 4-day bFF-treated responders (n=6) and the 8-day bFF-treated responders (n=6) (Groups 2a & 2b: lower panel) in Experiment 2. Day 0 = the day of synchronized oestrus. GnRH was given on Day 6 and PGF₂ α (PG3) was given on Day 9. The bars indicate the periods of bFF treatment.



comparison of 4-day and 8-day bFF-treatment (Groups 2a & 2b) on mean progesterone profiles is shown in the lower panel. Mean total progesterone production in the bFF-treated groups was increased when compared to the saline-treated group, but these differences were not statistically significant due to between-animal variation in serum progesterone concentrations.

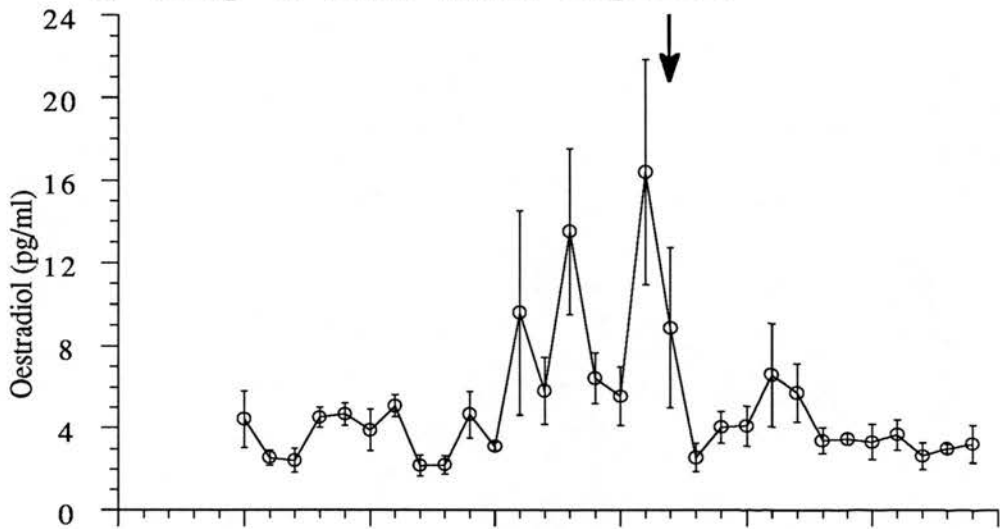
Retrospective re-analysis of individual progesterone profiles of the saline- and bFF-treated responders, in which rectal ultrasonography 6 days after GnRH had confirmed the presence of the induced CL, revealed that 13 (81%) of the GnRH responders had a functional CL as defined in section 4.2.5. Four (80%) of the saline-treated responders (Group 1f), 3 (60%) of the 4-day bFF-treated responders (Group 2af) and 6 (100%) of the 8-day bFF-treated responders (Group 2bf) had a functional induced CL. The mean (\pm sem) period of progesterone production from PG3, as defined in section 4.2.5., was 7.7 ± 0.5 days in Group 2bf, and extended by approximately 1.5 days when compared to Group 2af (6.3 ± 0.6 days) and Group 1f (6.3 ± 0.9 days), but these differences were not statistically significant. There was no difference in the mean (\pm sem) peak progesterone concentration in the interval from PG3 to oestrus in Groups 1f (2.4 ± 0.6 ng/ml), 2af (2.4 ± 0.4 ng/ml) and 2bf (2.9 ± 0.3 ng/ml).

4.3.2.6. Peripheral oestradiol concentrations

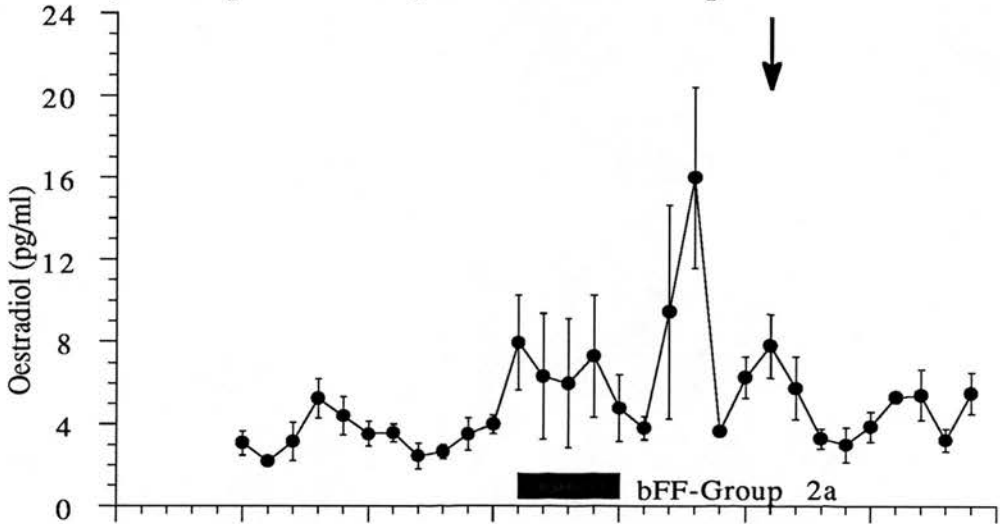
Mean serum oestradiol profiles for the saline-treated responders (Group 1), the 4-day bFF-treated responders (Group 2a) and the 8-day bFF-treated responders (Group 2b) are shown in Figure 4.10. Analysis of profiles preceding GnRH and following the subsequent oestrus reveal the expected early luteal phase concentrations of oestradiol (1-5 pg/ml), with a small rise associated with the growth of the first dominant follicle (Glencross *et al.*, 1973). However the treatment period (Days 5-12 after GnRH) was associated

Figure 4.10. Mean (\pm sem) serum oestradiol profiles for the saline-treated responders (Group 1; n=6: upper panel), 4-day bFF-treated responders (Group 2a; n=6: middle panel) and 8-day bFF-treated responders (Group 2b; n=6: lower panel) in Experiment 2. Day 0 = the day of synchronized oestrus. GnRH was given on Day 6 and PGF₂ α (PG3) was given on Day 9. Bars indicate the period of bFF treatment. Arrows indicate the mean onset of oestrus.

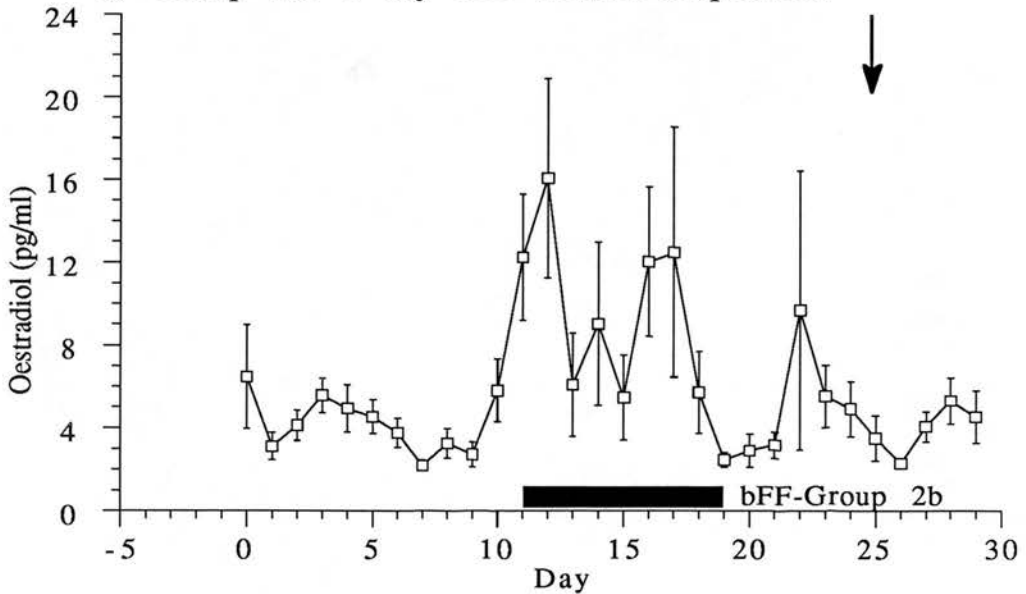
a) Group 1: Saline-treated responders



b) Group 2a: 4-day bFF-treated responders



c) Group 2b: 8-day bFF-treated responders



with higher concentrations of oestradiol in all 3 groups, although these differences were not statistically significant due to between animal variation within each group.

4.3.3. Experiment 3

Following the results of Experiment 2, the 8-day treatment period was repeated in Experiment 3 with an increased number of animals per group.

4.3.3.1. Response to GnRH

Twenty (91%) of the 22 heifers responded to the GnRH injection with an additional ovulation. The mean interval from PG3 to oestrus was extended by 3.9 days ($P < 0.01$) in the saline-treated responders (Group 1) compared to the non-responders and by 10.7 days ($P < 0.001$) in Group 2 (8-day bFF-treated responders) compared to the non-responders. Comparing the bFF-treated group (Group 2) to the saline-treated responders (Group 1), there was an extension in the interval from PG3 to oestrus of 6.8 days in Group 2 ($P < 0.001$).

4.3.3.2. Ovarian observations

Ultrasound observations were similar to Experiment 1 (see section 4.3.1.2.).

4.3.3.3. Peripheral progesterone concentrations

As in Experiments 1 and 2 (see sections 4.3.1.5. and 4.3.2.5.), analysis of progesterone profiles revealed that mean serum progesterone concentrations fell in all heifers with the regression of the endogenous CL following PG3, but the presence of an induced CL, confirmed by rectal ultrasonography 6 days after GnRH, was associated with the persistence of luteal function in both groups. Mean serum progesterone profiles for the

saline-treated responders (Group 1) and the bFF-treated responders (Group 2) are shown in Figure 4.11.

Retrospective re-analysis of individual progesterone profiles of the saline- and bFF-treated responders, in which rectal ultrasonography 6 days after GnRH had confirmed the presence of the induced CL, revealed that 12 (60%) of the GnRH responders had a functional CL as defined in section 4.2.5. Seven (64%) of the saline-treated responders (Group 1f) and 5 (56%) of the bFF-treated responders (Group 2f) had a functional induced CL. The mean (\pm sem) period of progesterone production from PG3, as defined in section 4.2.5., was 10.3 ± 2.3 days in Group 2f, and extended significantly ($P < 0.01$) by approximately 5.0 days when compared to Group 1f (5.6 ± 1.2 days). There was no significant difference in the mean (\pm sem) peak progesterone concentration in the interval from PG3 to oestrus in Groups 1f (1.8 ± 0.3 ng/ml) and 2f (2.0 ± 0.3 ng/ml).

4.3.3.4. Peripheral oestradiol concentrations

Mean serum oestradiol profiles for the saline-treated responders (Group 1) and the bFF-treated responders (Group 2) are shown in Figure 4.12. Analysis of profiles preceding GnRH in both groups and following the subsequent oestrus in the saline-treated group (data unavailable for Group 2 as sampling discontinued when all heifers in the bFF-treated group had shown oestrus) again reveal the expected early luteal phase concentrations of oestradiol (1-5 pg/ml; see section 4.3.2.6.). The mean total oestradiol production in the treatment period (Days 5-12 after GnRH) was significantly greater ($P < 0.01$) in the saline-treated group when compared to the bFF-treated group. Mean serum oestradiol concentrations for the 2 groups were significantly different ($P < 0.05$) on Day 14 (Day 8 after GnRH). A significant increase in oestradiol ($P < 0.05$) was observed on Day 17 (Day 11 after GnRH) in

Figure 4.11. Mean (\pm sem) serum progesterone profiles for the saline-treated responders (Group 1: n=11) and the 8-day bFF-treated responders (Group 2: n=9) in Experiment 3. Day 0 = the day of synchronized oestrus. GnRH was given on Day 6 and PGF₂ α (PG3) was given on Day 9. The bar indicates the period of bFF treatment.

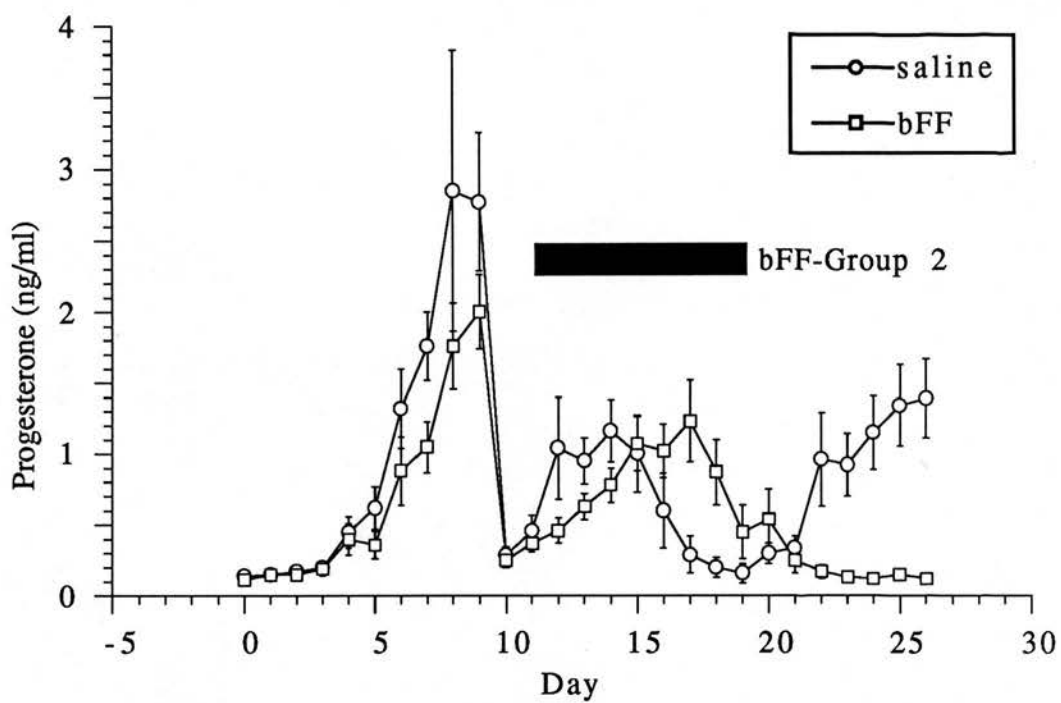
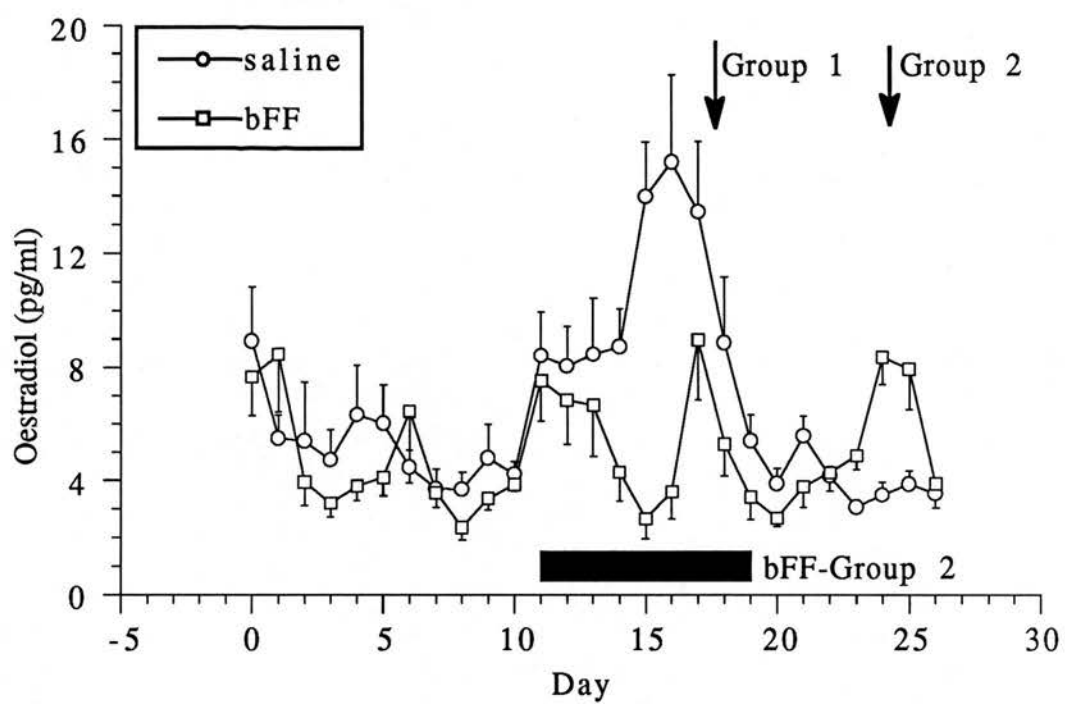


Figure 4.12. Mean (\pm sem) serum oestradiol profiles for the saline-treated responders (Group 1: n=11) and the bFF-treated responders (Group 2: n=9) in Experiment 3. Day 0 = the day of synchronized oestrus. GnRH was given on Day 6 and PGF_{2 α} (PG3) was given on Day 9. The bar indicates the period of bFF treatment. Arrows indicate the mean onset of oestrus.



the bFF-treated group before the cessation of bFF treatment, which was not associated with the presence of a dominant follicle: the dominant follicle did not emerge until after the cessation of bFF treatment.

4.4. Discussion

These studies have shown that there is a significant extension in the lifespan of the induced CL with the strategic administration of steroid-stripped bFF which suppresses the growth of the dominant follicle.

The administration of GnRH on Day 6 after the synchronized oestrus induced an additional ovulation in >70% ($86 \pm 4\%$: mean of Experiments 1, 2 and 3) of heifers confirming the observations in Chapter 3. There was an increase in the percentage of GnRH responders in Experiments 2 and 3, when compared to Experiment 1 and the study in Chapter 3. The same dose of GnRH (0.5 mg gonadorelin: based on the dose recommended by Intervet for the improvement of conception rate in the post-partum period) was used in all studies. All animals were sexually mature, but mean age and liveweight varied between studies. Mean (\pm sem) liveweights on the day of GnRH administration for heifers in Experiments 2 and 3, were 357.5 ± 5.5 kg and 433.2 ± 5.7 kg respectively. Liveweight data are unavailable for heifers in Experiment 1 and in the study in Chapter 3. However, heifers used in the preceding studies were older (>24 months of age), when compared to heifers used in Experiments 2 and 3 (12-18 months of age), and likely to have been significantly heavier. We were unable to carry out a dose-response study to test the hypothesis that the response to GnRH was related to bodyweight. There was no evidence for a breed effect on response rate to GnRH. Animals in Experiment 2 with higher response rate to GnRH were mixed continental and native crossbred heifers. All other heifers used in Experiment 3 with a

similar GnRH-response rate, and in the preceding studies with a lower response rate, were Hereford cross Friesian heifers.

The presence of the induced CL extended the period from PG3 to oestrus in the saline-treated responders in Experiments 1, 2 and 3, confirming the observations in Chapter 3, although the mean interval was reduced (3.9, 3.1 and 3.9 days respectively, compared to 4.7 days in the previous study in Chapter 3). Ultrasonography confirmed that oestrus coincided with the presence of a large dominant follicle on the ovaries. Administration of bFF significantly increased the period from PG3 to oestrus, when compared to the saline-treated responders, in all three experiments: 5.9 days (2.5-fold increase) in animals treated with bFF for 3 days; 4.4 days (2.4-fold increase) in animals treated for 4 days; and 7.4 days (mean of observations from Experiments 2 and 3: 3.1-fold increase) in animals treated for 8 days. However, irrespective of the time of onset and duration of bFF treatment, the interval from the cessation of bFF treatment to the subsequent oestrus was similar in all groups (Table 4.1.). A similar interval between the end of bFF treatment and the presence of a large (>10 mm diameter) ovulatory dominant follicle on the ovaries was observed by Wood, Glencross, Bleach, Lovell, Beard & Knight (1993), and corresponded approximately to the interval from emergence of the dominant follicle to ovulation, in heifers with 3 follicular waves (Ginther *et al.*, 1989a).

Table 4.1. Interval from the cessation of bFF administration to oestrus in Experiments 1, 2 and 3.

Experiment	Duration of bFF treatment (days)	Onset of bFF treatment (day after GnRH)	Interval from cessation of bFF to oestrus (days)
1	3	7	7.3 ± 0.4
2	4	5	7.0 ± 0.4
2	8	5	6.5 ± 0.2
3	8	5	6.2 ± 0.3

Treatment with bFF had no effect on LH concentrations. Mean concentrations of LH were similar in bFF-treated and saline-treated heifers. Injections of bFF did not decrease LH concentrations in ovariectomized rats (de Jong & Sharpe, 1976), ovariectomized heifers (Ireland, Curato & Wilson, 1983; Kiracofe, Ramirez-Godinez, McGowan & Bolt, 1983), intact heifers (Turzillo & Fortune, 1990), ovariectomized ewes (Cummins, O'Shea, Bindon, Lea & Findlay, 1983) and intact ewes (Miller, Critser & Ginther, 1982). There was no effect on LH pulse frequency and amplitude (sheep: McNeilly, 1984). In contrast, other workers in sheep have reported an increase in basal LH levels throughout treatment with bFF due to a significant increase in amplitude and frequency of pulsatile secretion (Wallace & McNeilly, 1985). *In vitro* studies using sheep pituitary cells have shown that preparations of inhibin (a component of follicular fluid) from pigs (Huang & Miller, 1984) and cattle (Muttukrishna & Knight, 1990), enhanced GnRH-induced LH release. In contrast, injections of follicular fluid *in vivo* did not affect GnRH-induced release of FSH or LH from bovine pituitary cells in culture (Hinshelwood, Kamel, Dierschke & Hauser, 1991).

Progesterone production by the induced CL was relatively low when compared with the spontaneously-formed CL in the early luteal phase (see Chapter 3, Figure 3.4.). However, mean concentrations of LH were similar in responders and non-responders, although Ireland & Roche (1982) reported that low levels of progesterone in the bovine female were associated with an increase in frequency of pulses of LH. A similar increase in LH pulsatility was reported in cows receiving subnormal levels of progesterone, indicative of luteal phase insufficiency (Roberson, Wolfe, Stumpf, Kittok & Kinder, 1989; Stock & Fortune, 1993). Mee, Stevenson, Alexander & Garth Sasser (1993) reported that administration of GnRH 12 hours after first detected oestrus, increased the average concentration of progesterone in the serum of pregnant and non-pregnant cows and decreased concentrations of LH, but had no effect on frequency of LH pulses on Days 1, 3 and 8 after oestrus. Although frequency and amplitude of LH secretion were not characterized in Experiments 1 and 2, our observations suggest that progesterone production by the induced CL was sufficient to maintain the negative feedback on gonadotrophin secretion (Ireland & Roche, 1982).

Treatment with bFF had no effect on FSH concentrations. The apparent reduction in mean serum FSH concentrations in the bFF-treated responders when compared to the saline-treated responders in Experiment 2 was unlikely to be an effect of the bFF treatment as there was a similar difference in the FSH profiles pre-GnRH administration. Bovine follicular fluid has been reported to suppress FSH concentrations when administered to ovariectomized rats (de Jong & Sharpe, 1976), ovariectomized cattle (Ireland *et al.*, 1983; Kiracofe *et al.*, 1983), intact sheep and cattle during the follicular phase of the oestrous cycle (cattle: Quirk & Fortune, 1986; Turzillo & Fortune, 1990; sheep: Miller *et al.*, 1982; McNeilly, 1984, 1985) and intact sheep during the luteal phase (Larson, Lewis, Dailey & Inskeep, 1985;

Wallace & McNeilly, 1985; Larson, Lewis, Dailey, Inskeep & Townsend, 1987). Other workers have reported no effect of follicular fluid on FSH secretion in ewes (Campbell, Picton, Mann, McNeilly & Baird, 1991) and heifers (Johnson & Smith, 1985; Law *et al.*, 1992). Uncertainty over whether bFF treatment suppresses FSH secretion in heifers is probably attributable to considerable inter-animal variation in plasma FSH, combined with the difficulty in distinguishing a bFF-induced fall in plasma FSH, in the follicular phase, from the spontaneous fall which occurs after luteolysis (Fortune, Sirois, Turzillo & Lavoie, 1991).

Cessation of bFF administration was associated with a significant increase in secretion of FSH in Experiment 2 (see Figure 4.8.). The increased secretion of FSH after the end of bFF treatment is a more consistent finding: a similar increase in FSH concentrations following cessation of follicular fluid treatment was observed in intact ewes (Miller *et al.*, 1982; McNeilly, 1984), intact heifers (Wood *et al.*, 1993), intact primates (Stouffer, Coensgen, di Zerega & Hodgen, 1981) and intact rats (de Paolo, Hirshfield, Anderson, Barraclough & Channing, 1979). The increase in secretion is attributable to the follicular fluid-induced suppression of follicular development leading to a temporary reduction in ovarian negative feedback (Campbell *et al.*, 1991). Studies involving ovariectomized heifers (Ireland *et al.*, 1983; Beard, Savva, Glencross, McLeod & Knight, 1989; Beard, Castillo, McLeod, Glencross & Knight, 1990) and ovariectomized ewes (Miller & Bolt, 1985) have shown that bFF can suppress FSH secretion in ovariectomized animals, although no FSH 'rebound' occurs after the end of treatment, confirming that the rebound effect is dependent on the presence of the ovaries.

FSH concentrations were not characterized for the non-responders in Experiments 1 and 2, but Mee *et al.*, (1993) reported that administration of

GnRH, 12 hours after first detected oestrus, increased average concentrations of FSH in serum and number of FSH pulses on Day 8.

In contrast to the observed lack of effect of bFF on gonadotrophin concentrations, the treatment period was associated with alterations in peripheral serum steroid concentrations. The induced CL secreted progesterone, again confirming the observations of Chapter 3, although the percentage of responders in which mean serum progesterone concentrations exceeded 1 ng/ml during the lifespan of the induced CL, was very variable (55%, 81% and 60% in Experiments 1, 2 and 3, respectively). Furthermore, the percentage of responders with a functional CL was not influenced by bFF treatment ($68 \pm 5\%$ and $67 \pm 11\%$ for the saline-treated responders and the bFF-treated responders respectively; means of Experiments 1, 2 and 3). The mean period of progesterone production and the mean peak progesterone concentration were generally increased in the bFF-treated responders, when compared to the saline-treated responders. However, there was no clear relationship between time of onset of bFF treatment and progesterone production, or between duration of bFF treatment and progesterone production (see Tables 4.2. and 4.3.). Indeed, from the progesterone profiles for the 8-day bFF-treated animals in Experiments 2 and 3, it is apparent that mean serum progesterone concentrations are declining before the cessation of bFF treatment (see Figures 4.9. and 4.11.).

Table 4.2. Increase in mean period of progesterone production in bFF-treated heifers when compared to saline-treated heifers.

Experiment	Duration of bFF treatment (days)	
	3	8
1	1.9 fold	-
2	-	1.2 fold
3	-	1.8 fold

Table 4.3. Increase in mean peak progesterone concentration in bFF-treated heifers when compared to saline-treated heifers.

Experiment	Duration of bFF treatment (days)	
	3	8
1	2.1 fold	-
2	-	1.2 fold
3	-	1.1 fold

In addition, bFF treatment was associated with differences in mean serum oestradiol concentrations. Ultrasonography confirmed that bFF suppressed the growth of the dominant follicle, and the presence of a novel factor(s) in bFF which acts to suppress follicular development directly has been demonstrated (Law *et al.*, 1992; Wood *et al.*, 1993). Thus, it was expected that serum oestradiol concentrations would be reduced in the treatment period because the dominant follicle is one of the major sources of peripherally measured oestradiol (Baird & Fraser, 1974; Zimmermann, Westhof, Thatcher, Peukert-Adam, Grunert & Braendle, 1990). Charcoal extraction removed approximately 98% of steroids (oestradiol and progesterone) from the follicular fluid. The concentrations of oestradiol and

progesterone in the follicular fluid after charcoal extraction were 7.6 and 15.2 ng/ml respectively. Assuming there was no clearance following an injection of 10 ml of bFF into a 400 kg heifer (blood volume = 40 litres), the circulating concentrations of oestradiol and progesterone would have been increased to 0.19 and 0.38 pg/ml respectively. These concentrations are lower than the oestradiol and progesterone concentrations in ovariectomized heifers (Beck, Smith, Seguin & Convey, 1976).

In Experiment 2, the lifespan of the induced CL was associated with increased concentrations of oestradiol (see Figure 4.10.). Similar concentrations were seen in the bFF-treated animals, suggesting that the effect was independent of the presence of a dominant follicle. It was hypothesized that the induced CL was contributing to its own demise by the secretion of luteolytic amounts of oestradiol. However, currently there is no evidence for the production of oestradiol by the bovine CL, unlike the rat and the rabbit (Rothchild, 1981). To investigate this hypothesis further, oestradiol content of induced luteal tissue was compared to oestradiol content of spontaneously-formed CL (see Chapter 5), and the experiment was repeated with an increased number of animals (Experiment 3) because of the large amount of variation between animals in Experiment 2. In Experiment 3, bFF-treatment significantly reduced serum oestradiol concentrations (see Figure 4.12.), implicating the follicle as the source of the raised oestradiol concentrations in the saline-treated responders. The reason for the increased peripheral oestradiol concentrations in Experiment 2 is therefore not known.

It is suggested that the effect of bFF on follicular development prevents the follicular phase rise in serum oestradiol associated with the emergence of a preovulatory follicle (Law *et al.*, 1992), so maintaining a luteal phase pattern of gonadotrophin secretion, and allowing the continued

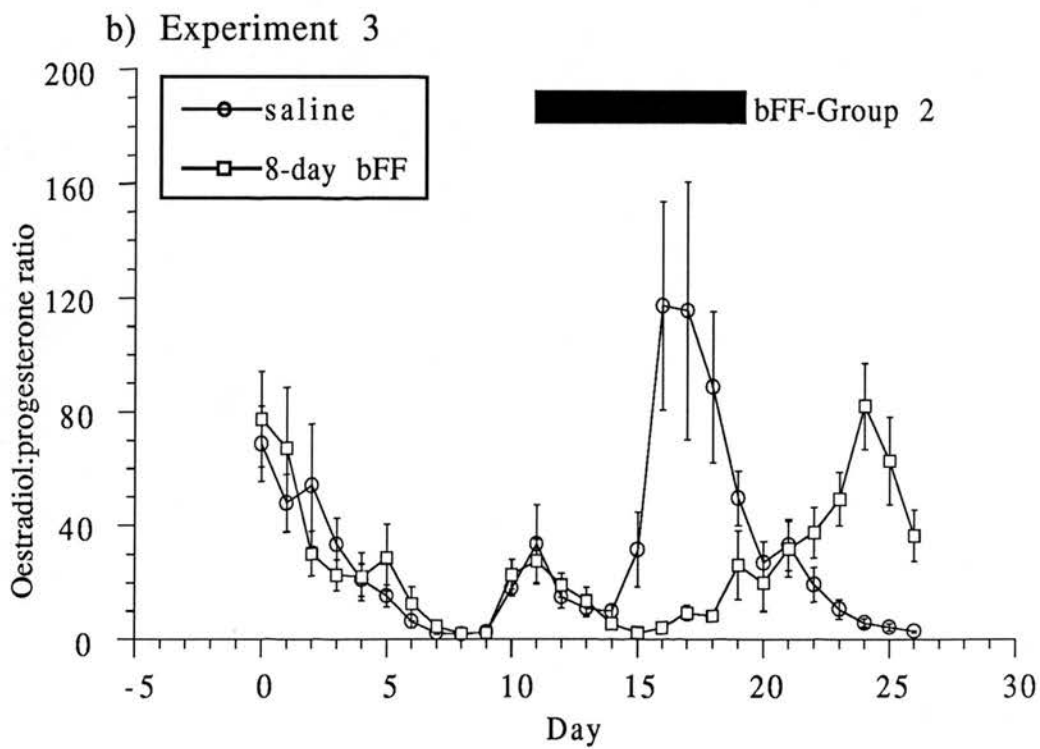
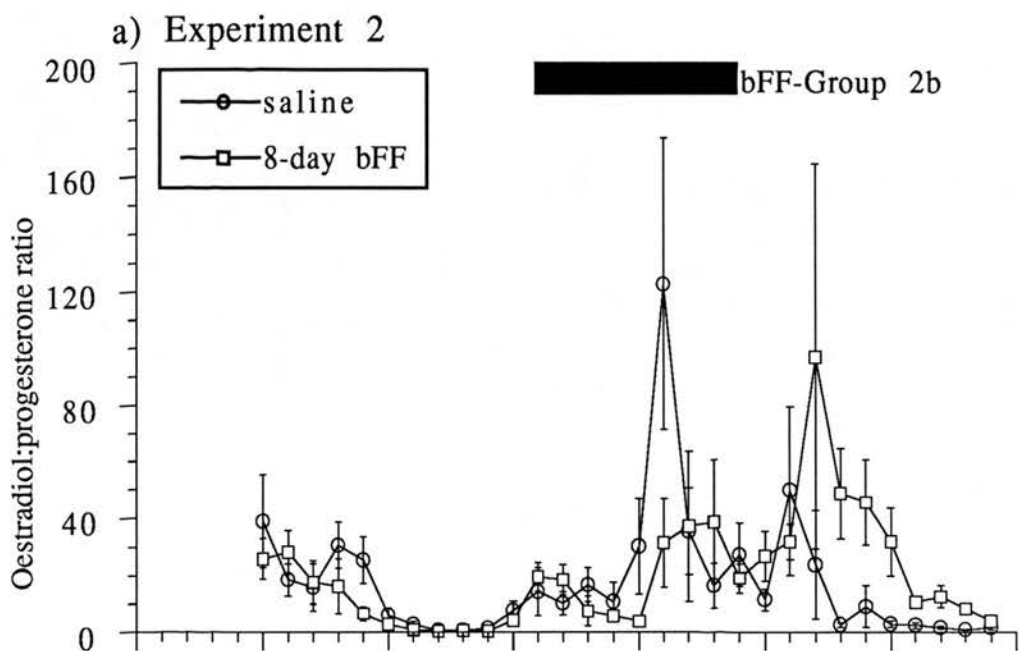
development of the induced CL. In Experiment 3, the decline in peripheral progesterone concentrations, which preceded the cessation of bFF treatment in the group treated for 8 days (see Figure 4.11.), coincided with an increased secretion of oestradiol on Day 17 (Day 11 after GnRH) (see Figure 4.12.), presumably derived from other steroidogenic sources such as smaller non-dominant follicles, atretic follicles, luteal tissue or peripheral sources such as fat tissue. Mean serum oestradiol concentrations on Day 17 in the bFF-treated group were similar to mean serum oestradiol concentrations at the subsequent oestrus (9.0 ± 2.1 pg/ml and 8.3 ± 1.0 pg/ml respectively), but although coincident with the regression of the induced CL, signs of behavioural oestrus were not observed. Normally, increased concentrations of plasma oestradiol in the presence of low levels of plasma progesterone, cause secretion of cervical mucus (Hansel, 1959), a manifestation of oestrous behaviour (Glencross, Esslemont, Bryant & Pope, 1981) and ovulation (Chenault, Thatcher, Kalra, Abrams & Wilcox, 1975) as a result of an LH surge (Short *et al.*, 1979; Peters, 1984).

As previously referred to in the Discussion, the percentage of responders with a functional CL was very variable (55%, 81% and 60% in Experiments 1, 2 and 3, respectively). It was hypothesized that in the heifers with a non-functional induced CL, oestrogen output by the large follicle exceeded a critical threshold value earlier in the lifespan of the induced CL and acted as a positive feedback agent, limiting progesterone production and peak progesterone concentration by initiating luteal regression. However, in Experiment 3 there was no difference in the mean total oestradiol production in the interval between administration of GnRH and the start of the treatment period, for the animals with a functional CL, when compared to the animals with a non-functional CL (30.0 ± 2.3 pg/ml and 25.7 ± 2.9 pg/ml respectively).

The possibility exists that other factors present in bFF can influence the lifespan of the induced CL (see Chapter 7). It seems unlikely that a luteotrophic factor present in the administered bFF is responsible for the delay in luteal regression, as spontaneous luteolysis is associated with the presence on the ovary of a growing follicle containing an increasing volume of follicular fluid. However a dose-dependent luteotrophic effect of the bFF cannot be eliminated.

Ovarian steroids (progesterone and oestradiol) can act as modulators of gonadotrophin secretion by working in concert during the bovine (Beck *et al.*, 1976; Stumpf, Roberson, Hamernik & Kittok, 1988b) and ovine (Karsch *et al.*, 1980) oestrous cycles. Furthermore, the interaction between progesterone and oestradiol influenced the regulation of the uterine oxytocin receptor in ovariectomized ewes (Zhang, Weston & Hixon, 1992). Profiles of the ratio of oestradiol to progesterone for the saline-treated responders compared to the 8-day bFF-treated responders in Experiments 2 and 3 are shown in Figure 4.13. There was a significant ($P < 0.01$) increase in the mean oestradiol:progesterone ratio, in the treatment period (Days 5-12 after GnRH) in Experiment 3. A similar increase was observed in Experiment 2, but this difference was not statistically significant due to between-animal variation within each group. Ratios of oestradiol to progesterone were similar in the first half of the treatment period, but differed with the onset of oestrus in the saline-treated group. Silvia & Taylor (1989) reported a positive relationship between the magnitude of the uterine PGFM response to oxytocin, which is part of the positive feedback loop that may drive the pulsatile release of $\text{PGF}_{2\alpha}$ during luteolysis, and the ratio of oestradiol to progesterone late in the oestrous cycle.

Figure 4.13. Mean (\pm sem) profiles of the oestradiol:progesterone ratio for the saline-treated responders (n=6) and the 8-day bFF-treated responders (n=6) (Groups 1 & 2b: upper panel) in Experiment 2, and the saline-treated responders (n=11) and the (8-day) bFF-treated responders (n=9) (Groups 1 & 2: lower panel) in Experiment 3. GnRH was given on Day 6 and PGF₂ α (PG3) was given on Day 9. The bars indicate the period of bFF treatment.



In conclusion, these studies have shown that administration of bFF during the development of the induced CL, suppresses the growth of the dominant follicle and associated with a further extension of luteal function. The results add further support to the conclusion of Chapter 3, that the CL induced by the administration of GnRH in the early luteal phase does not control its own lifespan, but rather, its demise appears to be associated with an endogenous signal from the dominant follicle. The bFF-induced alteration in lifespan of the induced CL is independent of changes in mean serum gonadotrophin concentrations. However, the bFF-induced reduction in mean serum oestradiol concentrations followed by the increase in oestradiol, coincident with the premature demise of the induced CL, provide circumstantial evidence that oestradiol is the endogenous agent responsible for the regression of the induced CL. In the absence of any significant differences in mean total oestradiol production, between heifers with a functional induced CL at the start of the treatment period and heifers in which the induced CL was non-functional, it is possible that differences in hypothalamo-pituitary sensitivity to the feedback effects of oestradiol, or differences between animals, in luteal and/or endometrial sensitivity to the luteolytic effect of oestradiol, determined whether the induced CL was functional or not.

CHAPTER 5

COMPARISON OF THE STRUCTURE AND COMPOSITION OF THE GnRH-INDUCED CORPUS LUTEUM WITH THE SPONTANEOUSLY-FORMED CORPUS LUTEUM

5.1.Introduction

The preceding studies have shown that the CL induced by the administration of GnRH in the early luteal phase does not control its own lifespan. Rather, its demise appears to be associated with an endogenous signal from the dominant follicle. Although luteal lifespan was extended by the strategic use of bFF to suppress the growth of the dominant follicle, during the development of the induced CL, the function of the induced CL was limited when compared to the spontaneous CL. The following criteria were selected as indices of luteal function: morphology, weight, progesterone content, oxytocin content, oestradiol content and LH receptor concentration.

Progesterone is the primary endocrine secretory product of the CL (Corner & Allen, 1929), and the luteal cell has the capacity for *de novo* steroidogenesis (Savard, 1973). However, the luteal cell has a limited capacity to store progesterone (Niswender & Nett, 1988), and so progesterone concentration can be used as an indicator of luteal function.

Concentration of oxytocin was also chosen as an indicator of functional competence of the developing CL, because the CL of cattle (Fields *et al.*, 1980; Wathes *et al.*, 1983) and sheep (Wathes & Swann, 1982) contain substantial quantities of oxytocin produced by *de novo* synthesis of the oxytocin-neurophysin precursor (Swann *et al.*, 1984).

Following the results of Experiment 2, Chapter 4 (see section 4.3.2.6.), it was hypothesized that the induced CL was contributing to its own demise by the secretion of luteolytic amounts of oestradiol because increased oestradiol concentrations were seen in animals in which follicular activity had been suppressed. Although there is no evidence for the presence of oestradiol in the bovine CL, oestradiol content of induced luteal tissue was compared to oestradiol content of spontaneously-formed CL to further investigate this hypothesis.

The CL in any species does not survive long in the absence of pituitary hormones (Rothchild, 1981). LH is the primary luteotrophic hormone in cattle, and LH maintains the structure of the gland and promotes progesterone secretion (Hansel *et al.*, 1973). Hormonal effects mediated via the interaction of LH with its membrane-bound receptor are dependent upon the following factors: (1) circulating LH concentration; (2) unoccupied LH receptor number; (3) the dissociation constant of the receptor for LH (Garverick *et al.*, 1985); (4) the integrity of the linkage of the LH receptor to second messenger generation; and (5) the sensitivity of the cell to respond to second messengers. Thus, LH receptor concentration is an index of the functional capacity of the CL. However, although the number of LH receptors was highly correlated with serum and luteal progesterone concentrations (Rao *et al.*, 1979; Spicer *et al.*, 1981; Garverick *et al.*, 1985), the biological significance of this correlation is unclear, as secretion of

progesterone is maximal when LH receptor number is highest but circulating concentrations of LH are lowest.

The aim of this study was to investigate further the reason for the premature demise of the GnRH-induced CL by a comparison of the structure and composition of the induced and spontaneously-formed CL. The age of the induced CL at the time of slaughter and collection of luteal tissue was Day 7, after administration of GnRH, because peripheral progesterone production is maximal at this time (see Chapter 3, Figure 3.4.). After Day 7 the premature regression of the induced CL was indicated by declining mean serum progesterone concentrations.

In addition, it is possible that $\text{PGF}_{2\alpha}$ given in the induced cycle, 3 days after GnRH, while not causing immediate regression of the induced CL, may partially compromise its function. To examine the effect of exogenous $\text{PGF}_{2\alpha}$ on luteal function, 7-day old spontaneous and induced luteal tissue that had been exposed to $\text{PGF}_{2\alpha}$ on Day 3 after administration of GnRH (Day 0), was collected.

5.2. Materials and Methods

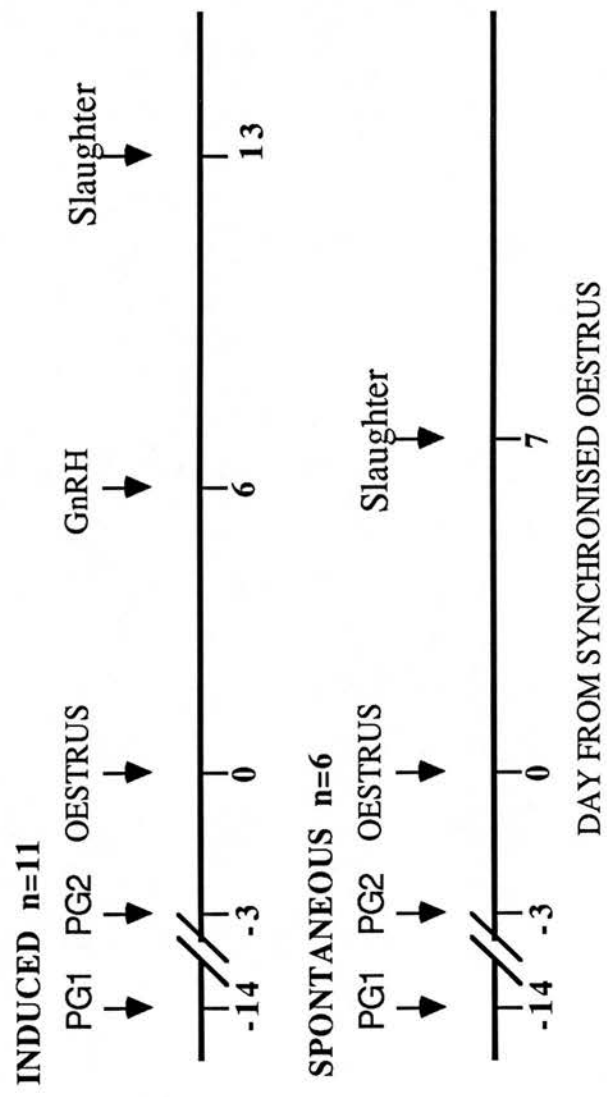
5.2.1. Animals

Mature Hereford cross Friesian heifers were housed in straw yards on the Institute Large Animal Unit and fed a maintenance ration of hay and grass nuts.

5.2.2. Experimental procedure

The protocol for Experiment 1 is shown in Figure 5.1. The oestrous cycles of 17 heifers were synchronized (oestrus = Day 0) with 2 injections of 500 ug cloprostenol (Estrumate: Coopers Animal Health, Crewe Hall,

Figure 5.1.PROTOCOL FOR EXPERIMENT 1



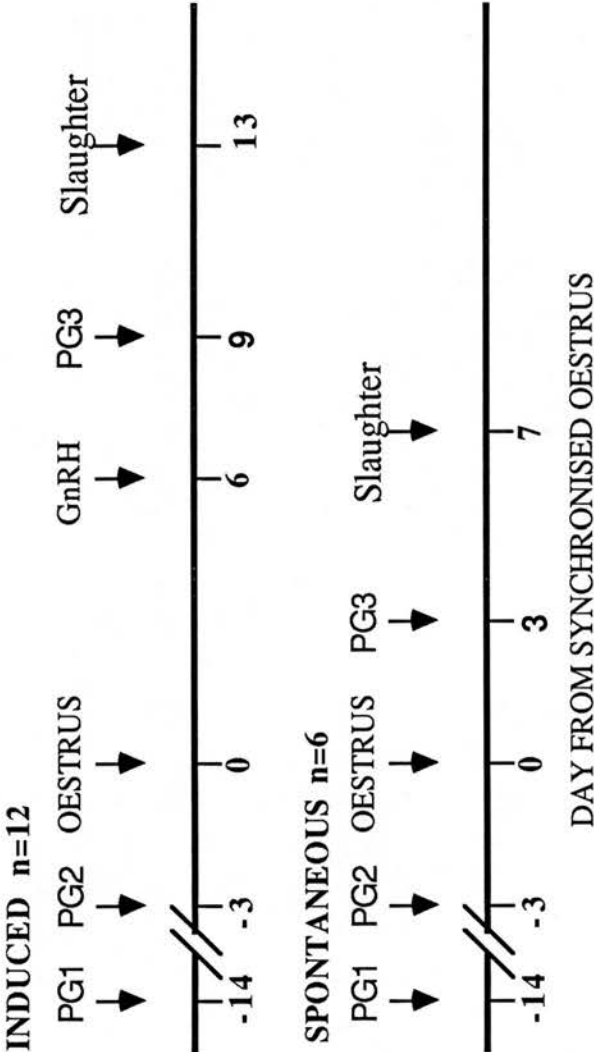
Cheshire), 11 days apart (PG1 and PG2). Oestrous behaviour following PG2 was monitored by 3 times-daily observation, together with the use of heat detection devices (Kamar Heatmount Detectors: Kamar Inc., Steamboat Springs, CO, USA). Oestrus was confirmed retrospectively by daily blood sampling for serum progesterone levels. Eleven heifers received 0.5 mg GnRH (GnRH; Fertagyl: Intervet Laboratories Ltd., Science Park, Milton Rd., Cambridge) by i.m. injection on Day 6 (oestrus = Day 0), and were slaughtered on Day 13. Six untreated heifers were slaughtered on Day 7. Ovaries were collected and photographed, all structures recorded and CL dissected, weighed and stored frozen (-80°C).

The protocol for Experiment 2 is shown in Figure 5.2. The oestrous cycles of 18 heifers were synchronized as before. Twelve heifers received 0.5 mg GnRH on Day 6, and 500 ug cloprostenol (PG3) on Day 9 (3 days after the induced ovulation), to remove the spontaneously-formed CL, and were slaughtered on Day 13. Three days after the synchronized oestrus (i.e. 3 days after the spontaneous ovulation), 6 heifers received 500 ug cloprostenol (PG3), when the spontaneously-formed CL would be expected to be non-responsive, and were slaughtered on Day 7. Luteal tissue was collected as before.

5.2.3. Hormone radioimmunoassays

Blood samples were collected by jugular venepuncture, and serum harvested and stored at -20°C for subsequent measurement of peripheral progesterone concentrations.

Figure 5.2.PROTOCOL FOR EXPERIMENT 2



5.2.3.1. Progesterone assay

Samples from Experiments 1 and 2 were assayed for progesterone in 1 assay. The sensitivity of the assay was 17.2 pg/tube (0.3 ng/ml) and the intra-assay coefficient of variation was 10.3%.

5.2.4. Tissue hormone content

5.2.4.1. Progesterone

Luteal tissue samples from Experiments 1 and 2 were assayed for progesterone in a single assay with a mean extraction efficiency of $51 \pm 1\%$. The sensitivity of the assay was 10.0 pg/tube and the intra-assay coefficient of variation was 11.2%.

5.2.4.2. Oestradiol

Luteal tissue samples from Experiments 1 and 2 were assayed for oestradiol in 3 assays with a mean extraction efficiency of $63 \pm 1\%$. The mean sensitivity of the assays was 0.7 pg/tube and the inter- and intra-assay coefficients of variation were 7.1% and 10.0% respectively.

5.2.4.3. Oxytocin

Luteal tissue samples from Experiments 1 and 2 were assayed for oxytocin in a single assay with a mean extraction efficiency of $65 \pm 2\%$. The sensitivity of the assay was 5.3 pg/tube and the intra-assay coefficient of variation was 9.2%. There was insufficient luteal tissue from heifers in Experiment 2 with spontaneously-formed CL exposed to $\text{PGF}_{2\alpha}$ on Day 3 (PG3) for determination of oxytocin content.

5.2.5. Tissue receptor content

5.2.5.1. LH

Luteal tissue samples from Experiments 1 and 2 were assayed for LH receptor in 2 assays. The inter- and intra-assay coefficients of variation were 13.1% and 8.2% respectively for 8 experiments in triplicate.

5.2.6. Statistical analysis

All data are presented as means \pm sem. Differences between means were tested by Student's *t* test.

Progesterone profiles were analyzed in the period between the spontaneous or GnRH-induced ovulation and slaughter. Differences between mean total progesterone production in the interval between administration of PG3 and slaughter were tested using Student's *t* test.

Luteal tissue collected at slaughter was not included in the statistical analysis from the following animals: heifers with evidence of non-response to the synchronisation regime (from retrospective analysis of progesterone profiles); heifers without an additional (induced) CL in response to the GnRH; heifers showing evidence of a double ovulation (2 CL); and heifers showing evidence of non-ovulation (presence of a luteinized follicle).

5.3. Results

5.3.1. Peripheral progesterone concentrations

Analysis of peripheral progesterone profiles for the individual animals indicated that 2 of the 17 heifers in Experiment 1, and 2 of the 18

heifers in Experiment 2, did not undergo luteolysis in response to the double prostaglandin synchronisation regime. Concentrations of progesterone in the peripheral serum of all other animals fell below 1 ng/ml following PG2.

Mean serum progesterone profiles for animals in Experiments 1 and 2 are shown in Figure 5.3. Concentrations of progesterone in the peripheral serum were elevated at the GnRH-induced ovulation due to the presence of the endogenous CL, and continued to increase until the day of slaughter (Day 13). However, following PG3 on Day 9 to remove the endogenous CL, mean serum progesterone profiles of animals with induced CL were similar to the profiles of animals with spontaneously-formed CL of the same age. Progesterone production in the interval between lysis of the endogenous CL with PG3 and slaughter, was similar for animals with an induced CL (6.3 ± 0.6 ng/ml) to animals with a spontaneously-formed CL (6.3 ± 0.4 ng/ml).

5.3.2. Response to GnRH

5.3.2.1. Experiment 1

Nine (82%) of the 11 treated heifers responded to the GnRH challenge with an additional ovulation.

5.3.2.2. Experiment 2

Nine (75%) of the 12 treated heifers responded to the GnRH challenge with an additional ovulation.

5.3.3. Gross morphology

Induced CL were indistinguishable morphologically from spontaneously-formed CL of the same age. Figure 5.4. shows the ovary of a GnRH responder with a 7-day old induced CL (upper plate), and the ovary of an untreated heifer with a 7-day old spontaneously-formed CL (lower plate).

Figure 5.3. Mean (\pm sem) serum progesterone profiles for the heifers with an additional GnRH-induced CL (treated with PG3: n=9; untreated: n=6), superimposed on mean (\pm sem) serum progesterone profiles for the heifers with a spontaneous CL (treated with PG3: n=4; untreated: n=3). Day 0 (Experiment 1) = day of synchronized oestrus for the GnRH-treated heifers and Day 0 (Experiment 2) = day of synchronized oestrus for the untreated heifers. GnRH was given on Day 6, and PGF₂ α (PG3: Experiment 2) was given on Day 9 (GnRH-treated heifers) and Day 3 (untreated heifers).

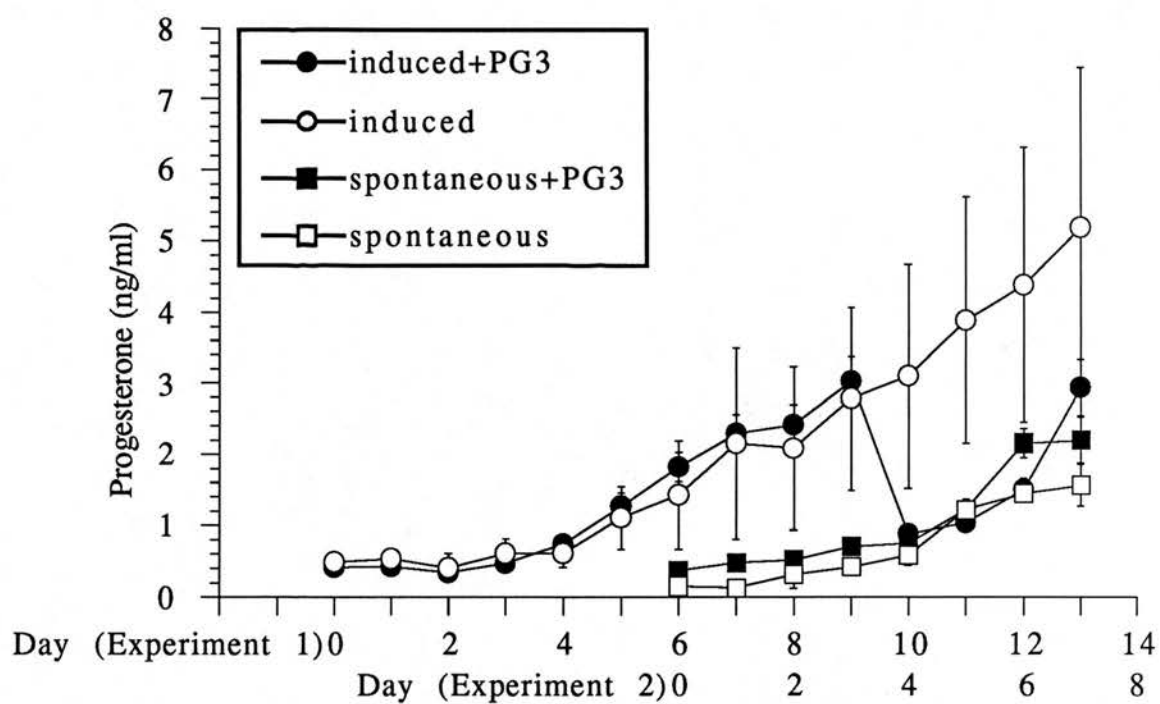


Figure 5.4. Photographs of the ovary of a GnRH responder with a 7-day old induced CL (upper plate), and the ovary of an untreated heifer with a 7-day old spontaneously-formed CL (lower plate).

Magnification of lower plate was x0.8 and magnification of upper plate was x1.6.



5.3.4. Luteal weight

Mean weights of spontaneously-formed and GnRH-induced CL of the same age are shown in Figure 5.5. Mean (\pm sem) weights of spontaneously-formed (3.9 ± 0.8 g) and induced CL (3.5 ± 0.4 g) of the same age were similar. Exposure of the induced CL to $\text{PGF}_{2\alpha}$ 3 days after the GnRH-induced ovulation resulted in a significant ($P < 0.01$) reduction in mean weight (2.6 ± 0.3 g) when compared to the CL exposed to $\text{PGF}_{2\alpha}$ 3 days after spontaneous ovulation (4.6 ± 0.6 g). However, mean weights of $\text{PGF}_{2\alpha}$ -exposed *versus* unexposed spontaneously-formed CL were not significantly different, and neither were mean weights of $\text{PGF}_{2\alpha}$ -exposed *versus* unexposed induced CL.

5.3.5. Tissue hormone content

5.3.5.1. Progesterone

Mean progesterone concentrations of spontaneously-formed and GnRH-induced CL of the same age are shown in Figure 5.6. Mean (\pm sem) concentrations of progesterone in induced CL (39.9 ± 4.0 $\mu\text{g/g}$ wet weight) and spontaneously-formed CL (42.1 ± 7.9 $\mu\text{g/g}$ wet weight) of the same age were similar. Furthermore, exposure of the spontaneously-formed and induced CL to $\text{PGF}_{2\alpha}$ 3 days after ovulation had no significant effect on the progesterone concentration on Day 7 (39.8 ± 2.0 $\mu\text{g/g}$ wet weight vs. 52.6 ± 11.0 $\mu\text{g/g}$ wet weight: spontaneous vs. induced respectively).

5.3.5.2. Oestradiol

No difference in oestradiol content was found between the induced or spontaneously-formed CL. Mean (\pm sem) concentrations for oestradiol in induced CL and spontaneously-formed CL were 1.2 ± 0.1 ng/g wet weight and 1.5 ± 0.1 ng/g wet weight respectively. For comparison the level of oestradiol

Figure 5.5. Mean (\pm sem) CL weights of spontaneously-formed and GnRH-induced CL of the same age. Grey columns represent CL not exposed to PGF₂ α (PG3) on Day 3 (Experiment 1; spontaneous: n=3; induced: n=6). Black columns represent CL exposed to PGF₂ α (PG3) on Day 3 (Experiment 2; spontaneous: n=4; induced: n=9). Means with similar letters are different (a: P<0.01).

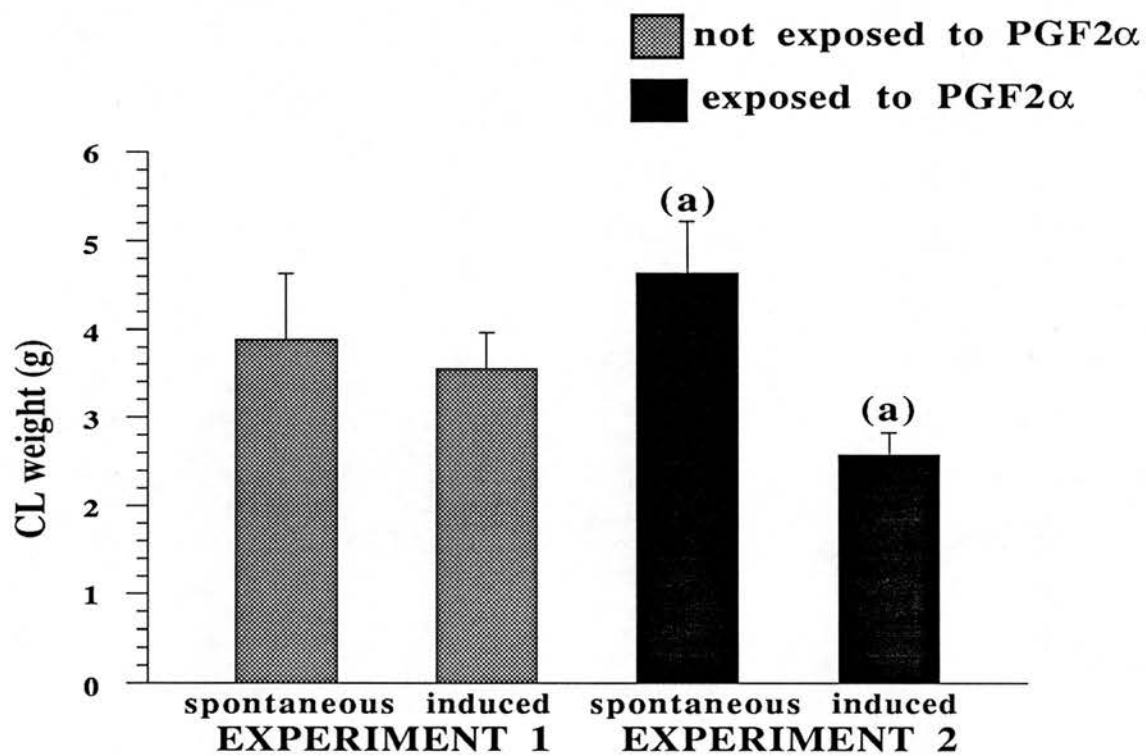
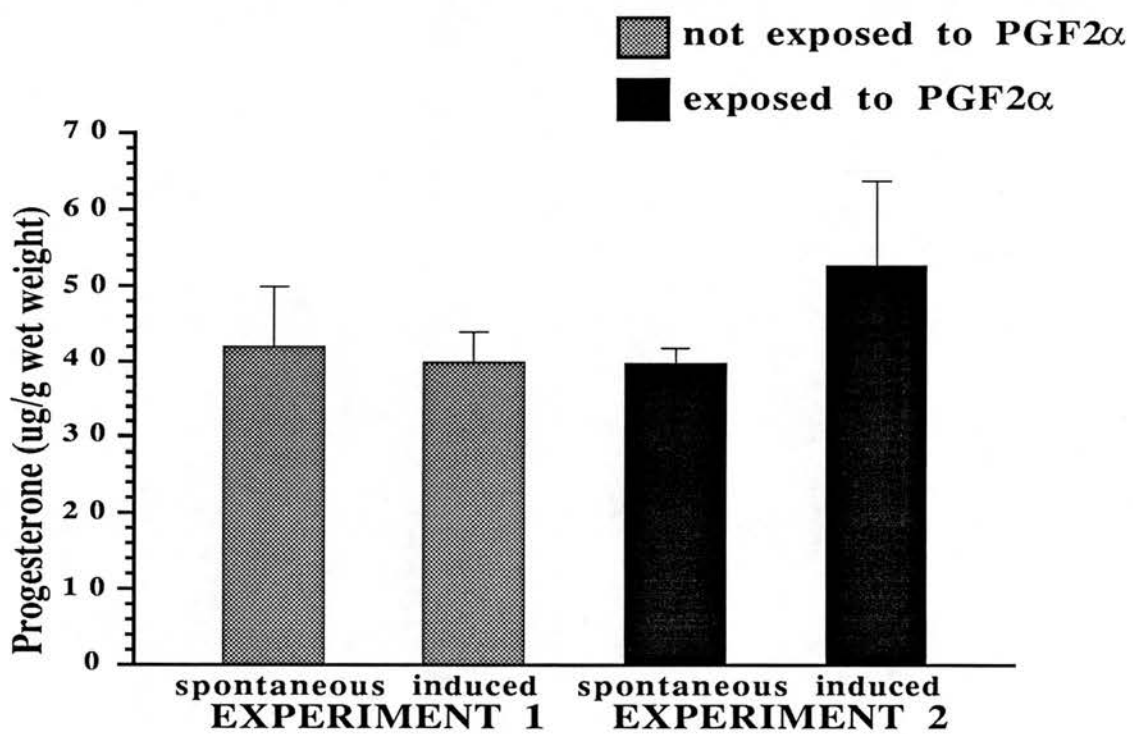


Figure 5.6. Mean (\pm sem) progesterone concentrations of spontaneously-formed and GnRH-induced CL of the same age. Grey columns represent CL not exposed to PGF₂ α (PG3) on Day 3 (Experiment 1; spontaneous: n=3; induced: n=6). Black columns represent CL exposed to PGF₂ α (PG3) on Day 3 (Experiment 2; spontaneous: n=4; induced: n=9).



in a non-ovulated follicle (obtained from a heifer which failed to ovulate in response to GnRH on Day 6) from which follicular fluid had been removed, was measured (13.0 ng/g wet weight).

5.3.5.3. Oxytocin

Mean (\pm sem) oxytocin concentrations of spontaneously-formed and GnRH-induced CL of the same age are shown in Figure 5.7. Concentrations of oxytocin in induced CL (535.6 ± 66.6 ng/g wet weight) and spontaneously-formed CL (460.6 ± 92.5 ng/g wet weight) of the same age were similar. Furthermore, exposure of the induced CL to PGF_{2 α} 3 days after ovulation had no significant effect on the oxytocin concentration on Day 7 (368.0 ± 47.8 ng/g wet weight) when compared to unexposed induced CL.

5.3.6. Tissue receptor content

5.3.6.1. LH

Mean LH receptor concentrations of spontaneously-formed and GnRH-induced CL of the same age are shown in Figure 5.8. LH receptor number was significantly increased ($P < 0.05$) in induced CL (37.7 ± 8.5 pg LH bound/mg protein) when compared to spontaneously-formed CL of the same age (13.0 ± 3.6 pg LH bound/mg protein). Exposure of the CL to PGF_{2 α} 3 days after ovulation resulted in a significant increase ($P < 0.01$) in LH receptor concentration in the 7-day old spontaneously-formed CL (46.5 ± 3.7 pg LH bound/mg protein), but had no effect on the LH receptor concentration in the GnRH-induced CL (36.4 ± 5.2 pg LH bound/mg protein).

5.4. Discussion

The comparison of 7-day old GnRH-induced CL and spontaneously-formed CL, has shown that: (1) mean CL weights of induced and spontaneous

Figure 5.7. Mean (\pm sem) oxytocin concentrations of spontaneously-formed and GnRH-induced CL of the same age. Grey columns represent CL not exposed to PGF₂ α (PG3) on Day 3 (Experiment 1; spontaneous: n=3; induced: n=6). The black column represents CL exposed to PGF₂ α (PG3) on Day 3 (Experiment 2; induced: n=9).

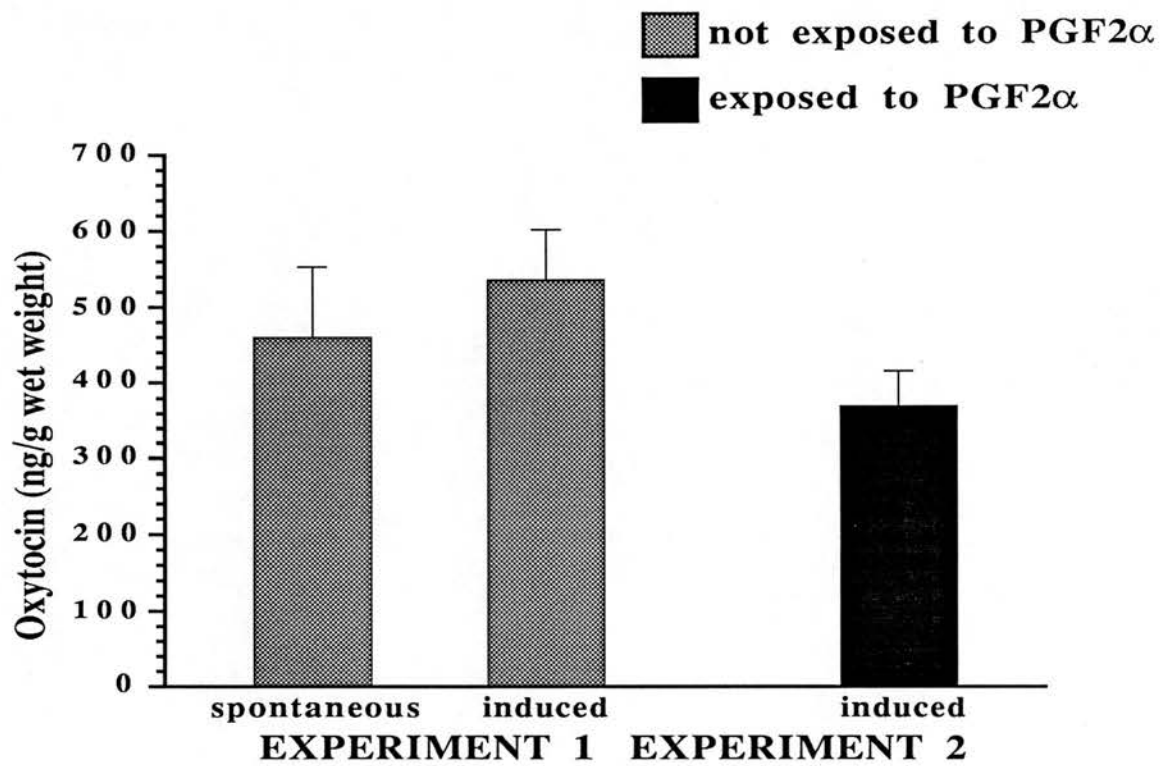
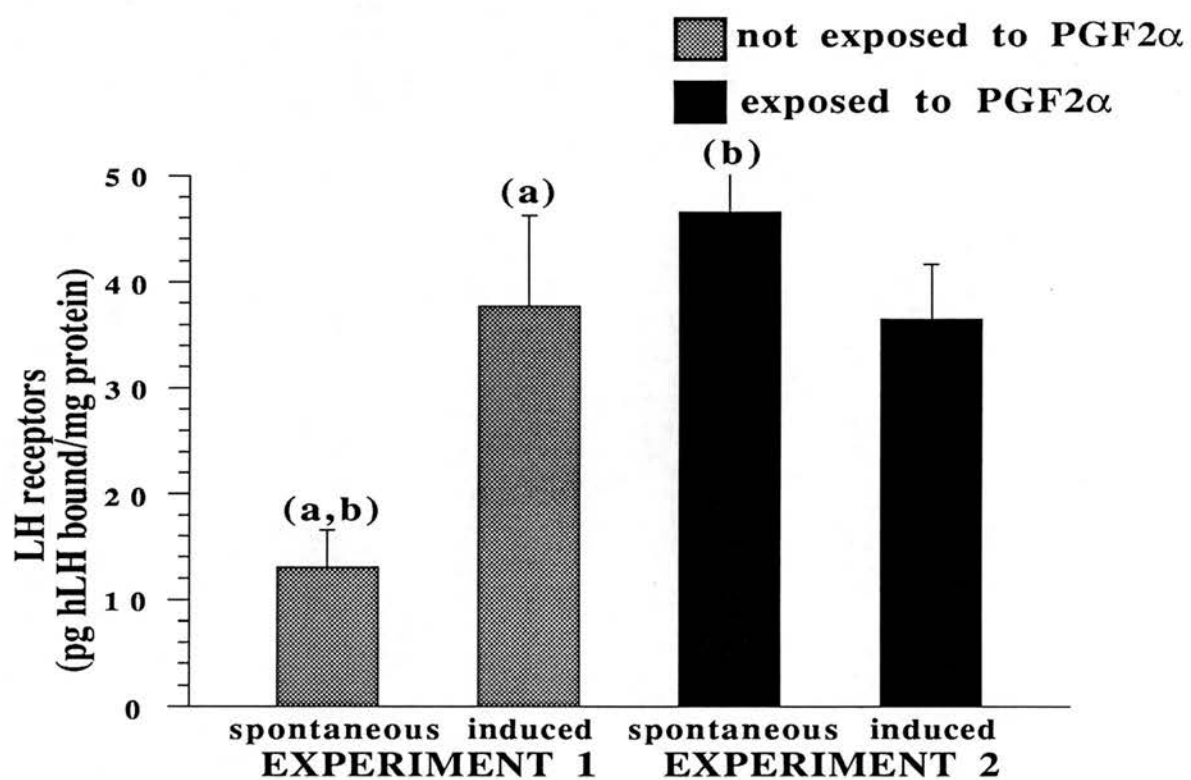


Figure 5.8. Mean (\pm sem) LH receptor concentrations of spontaneously-formed and GnRH-induced CL of the same age. Grey columns represent CL not exposed to PGF₂ α (PG3) on Day 3 (Experiment 1; spontaneous: n=3; induced: n=6). Black columns represent CL exposed to PGF₂ α (PG3) on Day 3 (Experiment 2; spontaneous: n=4; induced: n=9). Means with similar superscripts are different (a: P<0.05; b: P<0.01).



CL were not different; (2) tissue oxytocin and progesterone concentrations were similar; and (3) LH receptor concentration was increased in induced CL.

Induced and spontaneous CL of the same age were morphologically indistinguishable. Abnormal CL induced by GnRH treatment of anoestrous ewes (Hunter *et al.*, 1988) could not be distinguished from normal CL before Day 5 by measurement of progesterone in peripheral plasma. However, although the 2 types of CL were still indistinguishable on Day 4, differences were apparent macroscopically on Day 5, when abnormal CL were pale in appearance whereas normal CL remained dark pink in colour.

Although induced and spontaneous luteal weights were not different in this study, in a subsequent study (see Chapter 8, section 8.3.1.2.) mean weight of spontaneously-formed CL (6.5 ± 0.5 g) was significantly increased when compared to mean weight of induced CL (3.8 ± 0.5 g), which was similar to induced luteal weight in this study. Furthermore, mean CL weight was reduced in induced CL after exposure to $\text{PGF}_{2\alpha}$ on Day 3, when compared to similarly exposed spontaneously-formed CL. However, mean weights of induced CL exposed to $\text{PGF}_{2\alpha}$ and unexposed induced CL were not different, and mean weights of spontaneous CL exposed to $\text{PGF}_{2\alpha}$ and unexposed spontaneous CL were not different. Thus, the effect of exposure to exogenous $\text{PGF}_{2\alpha}$ 3 days after ovulation on luteal weight, appears not to be consistent between studies, and hence the results are difficult to interpret.

Luteal weight in post-partum beef cows, in which ovulation had been induced with hCG, did not differ between normal CL (pretreated with progesterone for 9 days) and subnormal CL, on Day 6 after ovulation (Hu, Sanders, Kurz, Ottobre & Day, 1990). Kesler *et al.* (1981a) reported that CL obtained from cows 5 days after ovulation following GnRH were similar in weight to CL removed from normally cycling cows on Day 5, although CL

were more than twice as large in oestrous cycle cows than in postpartum cows on Day 7. A divergence in plasma progesterone concentrations between the 2 groups was observed between Days 5 and 7, as the concentration of progesterone continued to increase to Day 7 in oestrous cycle cows, whereas concentrations declined in postpartum GnRH-treated cows. The 2.5-fold increase in CL weight and the rapid increase in progesterone concentrations during this period have also been reported by other investigators (Donaldson *et al.*, 1965; Erb, Randel & Callahan, 1971; Garverick, Erb, Niswender & Callahan, 1971). In this study, peripheral progesterone concentrations continued to increase between Days 5 and 7 after both spontaneous and GnRH-induced ovulation (see Figure 5.3.), following exposure to PGF₂ α on Day 3. A comparison of progesterone profiles between animals with spontaneous and induced CL, but unexposed to PG3 in a similar period, was not possible due to the presence of the endogenous CL in the GnRH responders (Rajamahendran & Sianangama, 1992).

Oxytocin concentrations in induced and spontaneous CL were similar. Furthermore, oxytocin concentrations were not significantly reduced in PGF₂ α -exposed induced CL. In both cows and ewes, luteal oxytocin content increased to a maximum in the mid-luteal phase and then declined (Sheldrick & Flint, 1983b; Fehr, Ivell, Koll, Schams, Fields & Richter, 1987; Jones & Flint, 1988; Parkinson, Wathes, Jenner & Lamming, 1992). The oxytocin concentration decreased before the fall in progesterone concentration, and therefore the main decrease in oxytocin concentration preceded the onset of luteolysis (Wathes *et al.*, 1984). Concentrations of oxytocin increased in CL of cycling cattle until the mid-luteal phase (Days 5-7: 199 ± 43 ; Days 8-13: 447 ± 93 ng/g wet weight) and decreased afterwards (Schams *et al.*, 1985a). Thus, the anticipated premature demise of the induced

CL was not preceded by reduced luteal oxytocin concentrations on Day 7 after GnRH administration.

Luteal progesterone concentrations in spontaneous and induced CL were similar. Hunter *et al.* (1988) reported a significant decrease in progesterone content, 5 days after ovulation, of abnormal CL induced by GnRH treatment of anoestrous ewes. In contrast, progesterone concentration on Day 5 and secretion of progesterone from oestrus to Day 5, were similar in post-partum beef cows anticipated to have a short or normal oestrous cycle (Smith *et al.*, 1986). Furthermore, Rutter *et al.* (1985) reported similar luteal progesterone concentrations in oestrous cycle and post-partum cows 7 days after ovulation. In this study, progesterone concentrations in spontaneously-formed and induced CL were unaffected by exposure to PGF_{2α}. Furthermore, progesterone secretion by PGF_{2α}-exposed spontaneous CL was similar to unexposed CL, although Helmer & Britt (1987) reported that progesterone secretion during the first week of the oestrous cycle was lower in heifers given 25 mg PGF_{2α} on Day 3, with repeated doses 12 and 24 hours later, than in saline-treated animals. Additionally, progesterone secretion by the induced CL, following regression of the endogenous CL, was similar to the spontaneously-formed CL of the same age.

The number of LH receptors was reduced in spontaneous CL when compared to induced CL (Figure 5.8.). Jones, Ottobre & Pate (1992) reported that the number of LH receptors per cell was approximately 3 times as high on cultured cells from mid-cycle CL as it was on cultured cells from early CL. Furthermore, an increase in the number of receptors for LH from Days 6-10, without subsequent changes on Day 15 of the oestrous cycle, was observed by Harrison *et al.* (1987), suggesting that by mid-cycle luteal cells have attained their maximum complement of receptors for LH. A similar increase in the concentration of unoccupied LH receptors during luteal development was

reported by Garverick *et al.* (1985), and highly correlated with plasma progesterone concentrations. Unoccupied receptor concentrations were doubled on Day 7 and quadrupled on Day 10 to 16 compared with Day 4. It is possible that progesterone production by the endogenous CL was responsible for the premature induction of LH receptors in the GnRH responders. Exposure of cultured bovine luteal cells to an increasing concentration of exogenous progesterone resulted in an elevation of receptor numbers in cells from early, but not mid-cycle, CL (Jones *et al.*, 1992). The ability of progesterone to elevate LH receptor numbers in early CL may be an example of its luteotrophic nature as proposed by Rothchild (1981). In contrast, CL from cycling cows had a higher LH receptor concentration than post-partum cows 7 days after GnRH administration (Rutter *et al.*, 1985). Studies in cattle and sheep with CL anticipated to have short lifespans have shown no early decrease in LH/hCG binding or adenylate cyclase activity (Smith *et al.*, 1986; Hunter *et al.*, 1988), although LH receptor content did not exhibit the developmental increase measured in the normal CL on Day 5 after GnRH-induced ovulation in ewes (Hunter *et al.*, 1988). In this study, both the reason for the observed increase in LH receptor concentration in the induced CL and the biological significance are unclear.

Exposure of the spontaneous CL to $\text{PGF}_{2\alpha}$ on Day 3 after ovulation increased LH receptors on Day 7 to concentrations similar to those found in the induced CL. The bovine CL is unresponsive to the luteolytic effects of $\text{PGF}_{2\alpha}$ in the first 5 days after ovulation. However $\text{PGF}_{2\alpha}$ may cause an earlier induction of LH receptors or delay the transformation of (LH receptor-rich) small luteal cells into (LH receptor-poor) large luteal cells (Fitz *et al.*, 1981). The rapid inhibitory effect of $\text{PGF}_{2\alpha}$ in the $\text{PGF}_{2\alpha}$ -responsive phase of the oestrous cycle, operates via a mechanism not mediated through interference with gonadotrophin binding (Thomas *et al.*, 1978).

In conclusion, these results demonstrate there was no difference between spontaneous and induced CL in progesterone, oxytocin and oestradiol content and suggest that other factors are involved in the mechanism(s) limiting the lifespan of the induced CL. Furthermore, the absence of a decrease in LH binding indicates that the short lifespan of the induced CL is not a consequence of the lack of luteotrophic support. This study has examined specific characteristics of luteal function in isolation. Further investigation will be directed at an assessment of luteal function *in vivo* by examining the interaction between the induced CL and the uterine endometrium.

CHAPTER 6

INVESTIGATION OF THE EFFECT OF AN OXYTOCIN CHALLENGE ON THE PROSTAGLANDIN RESPONSE IN HEIFERS WITH A GnRH-INDUCED CORPUS LUTEUM

6.1.Introduction

In non-pregnant ruminants the regression of the CL is thought to result from an interaction between oxytocin, released by the CL, and the uterine endometrium, resulting in the pulsatile release of $\text{PGF}_{2\alpha}$ (Ginther *et al.*, 1967; Milvae & Hansel, 1980b; Schams, Walters, Schallenberger, Bullerman & Karg, 1983b; Oyedipe *et al.*, 1984; Wathes, 1984; Lafrance & Goff, 1985). Pulses of oxytocin (and its associated neurophysin) occur concurrently with pulses of $\text{PGF}_{2\alpha}$ during luteolysis (Webb *et al.*, 1981b; Flint & Sheldrick, 1983; Vighio & Liptrap, 1986). Oxytocin stimulates secretion of $\text{PGF}_{2\alpha}$ from the uterus (Newcomb *et al.*, 1977; Milvae & Hansel, 1980b; Oyedipe *et al.*, 1984). $\text{PGF}_{2\alpha}$ stimulates secretion of luteal oxytocin (Schallenberger *et al.*, 1984). Flint *et al.* (1986) have proposed that uterine $\text{PGF}_{2\alpha}$ and luteal oxytocin comprise a positive feedback loop. However the factors that initiate and terminate the loop have not been determined.

Uterine secretory responsiveness to oxytocin varies with the stage of the oestrous cycle. Oxytocin-stimulated $\text{PGF}_{2\alpha}$ release is maximal in the late luteal phase and responsiveness is maintained into the early part of the next

oestrous cycle (Roberts *et al.*, 1976; Fairclough *et al.*, 1984; Silvia & Taylor, 1989). The ovarian steroids, progesterone and oestradiol, contribute to the regulation of uterine secretory responsiveness to oxytocin (Lafrance & Goff, 1988). Oxytocin can stimulate uterine secretion of $\text{PGF}_{2\alpha}$ in ovariectomized ewes or cows only after the animal has been exposed to progesterone for 7-10 days (Homanics & Silvia, 1988; Lafrance & Goff, 1988). Furthermore, the ability of oxytocin to stimulate $\text{PGF}_{2\alpha}$ release from ovariectomized animals receiving progesterone is enhanced by treatment with oestradiol (McCracken, 1980; Homanics & Silvia, 1988; Lafrance & Goff, 1988; Vallet *et al.*, 1990; Raw & Silvia, 1991).

The aim of this experiment was to determine whether animals with a 7-day old GnRH-induced CL could respond to an oxytocin challenge with the release of $\text{PGF}_{2\alpha}$ (measured as PGFM, the primary metabolite of $\text{PGF}_{2\alpha}$, in the peripheral circulation). Also, in responding animals, to compare the magnitude of the PGFM response with that in control animals with a 7-day old spontaneously-formed CL, challenged with oxytocin. In addition, we determined whether the PGFM response in oxytocin-treated animals could be related to differences in the ovarian steroid environment during the period prior to the injection.

6.2. Materials and Methods

6.2.1. Animals

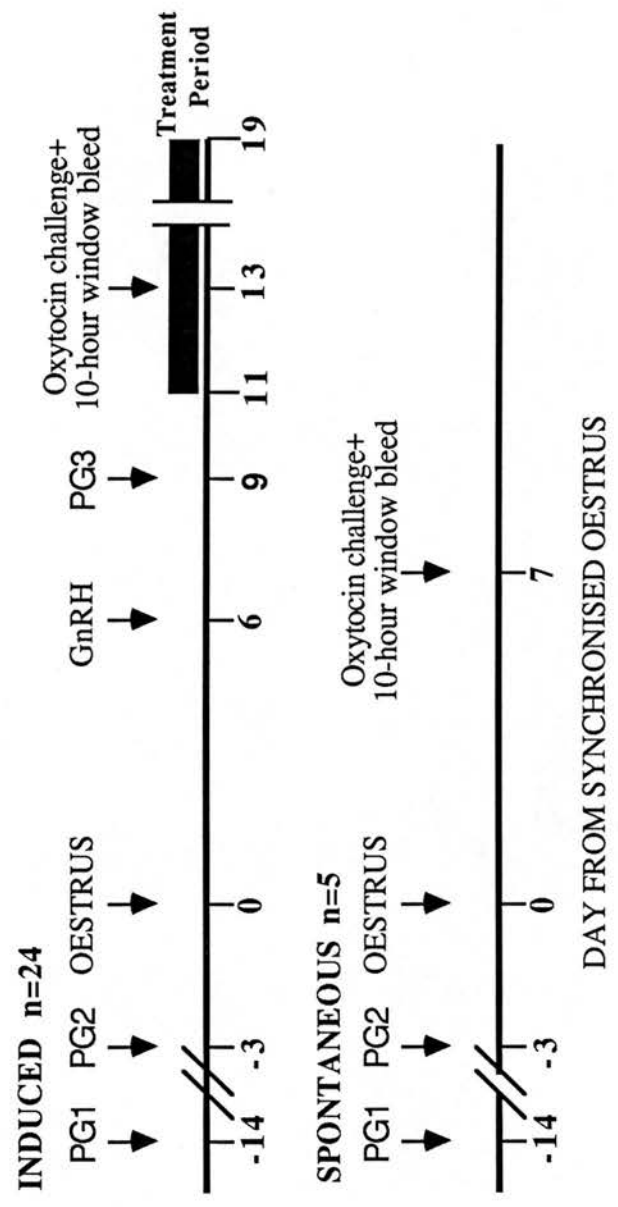
Mature Hereford cross Friesian heifers were housed in straw yards on the Institute Large Animal Unit and fed a maintenance ration of hay and grass nuts.

6.2.2. Experimental procedure

The experimental protocol is shown in Figure 6.1. and was initially similar to the procedure described in Chapter 3 (see section 3.2.2.). Briefly the oestrous cycles of 29 heifers were synchronized with 2 injections of 500 µg cloprostenol (Estrumate: Coopers Animal Health, Crewe Hall, Cheshire), 11 days apart (PG1 and PG2). Oestrous behaviour following PG2 was monitored by 3 times-daily observation, together with the use of heat detection devices (Kamar Heatmount Detectors: Kamar Inc., Steamboat Springs, CO, USA). Oestrus was confirmed retrospectively by daily blood sampling for serum progesterone levels. The heifers were randomised into 2 groups: Group A (n=5) and Group B (n=24). Seven days after the synchronized oestrus (oestrus = Day 0) Group A (control) received 0.33 IU oxytocin/kg (Oxytocin Leo: Leo Laboratories Ltd., Princes Risborough, Buckinghamshire) in 0.9% saline (20 IU oxytocin/ml) by subcutaneous (s.c.) injection (Milvae & Hansel, 1980b; Tsang *et al.*, 1990). A 10-hour window bleed (samples every hour: based on the results of Silvia & Taylor, 1989) was commenced 3 hours before the oxytocin challenge to characterise the PGFM response.

Six days after the synchronized oestrus (oestrus = Day 0) Group B heifers received 0.5 mg gonadorelin (Fertagyl: Intervet Laboratories Ltd., Science Park, Milton Rd., Cambridge) by i.m. injection. In preparation for laparoscopy food was withheld for 48 hours and water was withheld for 24 hours. All heifers were subjected to sublumbar laparoscopy 2 days after the gonadorelin injection. Animals with no new ovulation point were classified as non-responders. Heifers with a fresh ovulation point, and hence a new induced CL, were classified as GnRH responders (subsequent cycle referred to as the induced cycle, where day of GnRH injection = Day 0). Twenty-four

Figure 6.1.PROTOCOL FOR EXPERIMENT



hours after laparoscopy and 3 days after GnRH administration, all heifers received 500 µg cloprostenol (PG3) to remove the spontaneously-formed CL. Twenty-two heifers with an induced CL (responders) were split into 2 groups between Days 5-12 (the treatment period) after GnRH: Group B1 (saline-treated responders; n=12) received 10 ml 0.9% saline i-v 3 x daily for 8 days; Group B2 (bFF-treated responders; n=10) received 10 ml bFF i-v 3 x daily for 8 days. The non-responders (n=2) were discarded from the experiment. Animals were monitored by rectal ultrasonography on Day 6 after GnRH to confirm the presence or absence of the induced CL.

Seven days after the GnRH injection 6 heifers in Group B1 (50%) and 5 heifers in Group B2 (50%) received 0.33 IU oxytocin/kg in 0.9% saline (20 IU/ml) by s.c. injection. The other heifers in Groups B1 and B2 were untreated. A 10-hour window bleed (samples every hour) was commenced 3 hours before the oxytocin challenge to characterise the PGFM response. Oestrous behaviour was monitored as before by 3 times-daily observation, together with the use of Kamars, and confirmed retrospectively by daily blood sampling for serum progesterone levels.

6.2.3. Hormone radioimmunoassays

Blood samples for progesterone and oestradiol were collected by jugular venepuncture, and serum harvested and stored at -20°C for subsequent measurement of peripheral progesterone and oestradiol concentrations.

Blood samples for PGFM were collected by jugular venepuncture into heparinised tubes on ice, and plasma harvested and stored at -20°C for subsequent measurement of peripheral PGFM concentrations.

6.2.3.1. Progesterone assay

Samples from this study were assayed for progesterone in 3 assays. The mean sensitivity of the assays was 7.5 pg/tube (0.2 ng/ml) and the inter- and intra-assay coefficients of variation were 13.4% and 9.0% respectively.

6.2.3.2. Oestradiol assay

Samples from this study were assayed for oestradiol in 6 assays with a mean extraction efficiency of $55 \pm 1\%$. The mean sensitivity of the assays was 0.8 pg/tube and the inter- and intra-assay coefficients of variation were 14.8% and 12.5% respectively.

6.2.3.3. PGFM assay

Samples from this study were assayed for PGFM in 5 assays. The sensitivity of the assays was 2 pg/ml and the inter- and intra-assay coefficients of variation were 6.0% and 3.0% respectively.

6.2.4. bFF preparation

bFF was prepared as described in Chapter 2 (see section 2.7.1.). Dextran-coated charcoal treatment reduced concentrations of oestradiol and progesterone in the follicular fluid from 235.1 to 4.7 ng/ml and from 357.3 to 3.7 ng/ml, respectively.

6.2.5. Statistical analysis

Pretreatment concentrations of progesterone and oestradiol were defined as the concentration of each hormone in the sample collected 3 hours before the oxytocin challenge. The ratio of oestradiol to progesterone

was calculated for each heifer. Total progesterone was defined as the sum of the daily progesterone concentrations in the specified period.

The baseline concentration of PGFM was defined as the average concentration in the 4 samples collected at 1-hour intervals during the 4-hour period immediately prior to injection of oxytocin. The magnitude of the response was defined as the maximum concentration of PGFM observed during the first 6 hours post-injection, and was expressed as a percentage of the baseline PGFM concentration. The time to maximum response was defined as the time interval between the oxytocin challenge and the maximum concentration of PGFM measured in the 6 hours post-injection. The difference in time to maximum response between controls and responders was tested using Student's *t* test. PGFM profiles were compared using linear regression analysis on Genstat. The total amount of PGFM secreted was determined from the area under the curve.

and oestradiol, the ratio of oestradiol:progesterone, and the PGFM response were determined by linear regression analysis. In addition, the relationship between total progesterone secretion in the 7-day interval between ovulation and oxytocin injection for both controls and responders with the PGFM response, and that between progesterone secretion and PGFM response in the 4-day interval between PG3 and oxytocin injection for responders and the corresponding 4-day period preceding oxytocin injection for controls, were determined by linear regression analysis.

All data are presented as means \pm sem. The significance of differences between oxytocin-treated and untreated saline-treated responders and between oxytocin-treated and untreated bFF-treated responders in the interval from oxytocin to oestrus, and total progesterone production over a the same period, were tested by Student's *t* test.

6.3.Results

(i) *Baseline PGFM*

There was no difference between mean (\pm sem) baseline PGFM concentrations for each of the 3 groups (Group A: 113.0 ± 31.1 pg/ml; Group B1: 121.3 ± 25.6 pg/ml; Group B2: 119.5 ± 26.5 pg/ml).

(ii) *PGFM response (Groups A and B)*

The mean (\pm sem) PGFM response profiles for the controls (Figure 6.2.a) and the GnRH responders (Figure 6.2.b) are compared in Figure 6.2.c. There was no significant difference in the amount of PGFM secreted between the 2 groups.

The mean (\pm sem) magnitude of the oxytocin-stimulated PGFM response in the control animals ($76.3 \pm 33.7\%$) and GnRH responders ($58.3 \pm 13.6\%$) was not statistically significant due to between animal variation.

(iii) *Time to maximum PGFM concentration*

The times to maximum PGFM concentration for all oxytocin-treated heifers are shown in Figure 6.3. Nine heifers (56%) had a maximum response 1 hour post-oxytocin injection. Eight of these animals were GnRH responders. There was a significant increase ($P < 0.05$) in the mean time to maximum response in controls (2.0 ± 0.6 hours) compared to responders (1.3 ± 0.5 hours).

(iv) *PGFM response (Groups B1 and B2)*

The mean (\pm sem) PGFM response profiles for the saline-treated responders (Figure 6.4.a) and the bFF-treated responders (Figure 6.4.b) are

Figure 6.2. Mean (\pm sem) PGFM profiles in the 6-hour period following an oxytocin challenge, expressed as a percentage of mean PGFM concentration in the 4-hour prechallenge period. a) oxytocin-treated controls (n=5) with a spontaneous CL; b) oxytocin-treated (n=11) and untreated (n=11) GnRH responders with an induced CL; and c) comparison of oxytocin-treated controls and oxytocin-treated GnRH responders. Time 0 = time of oxytocin injection.

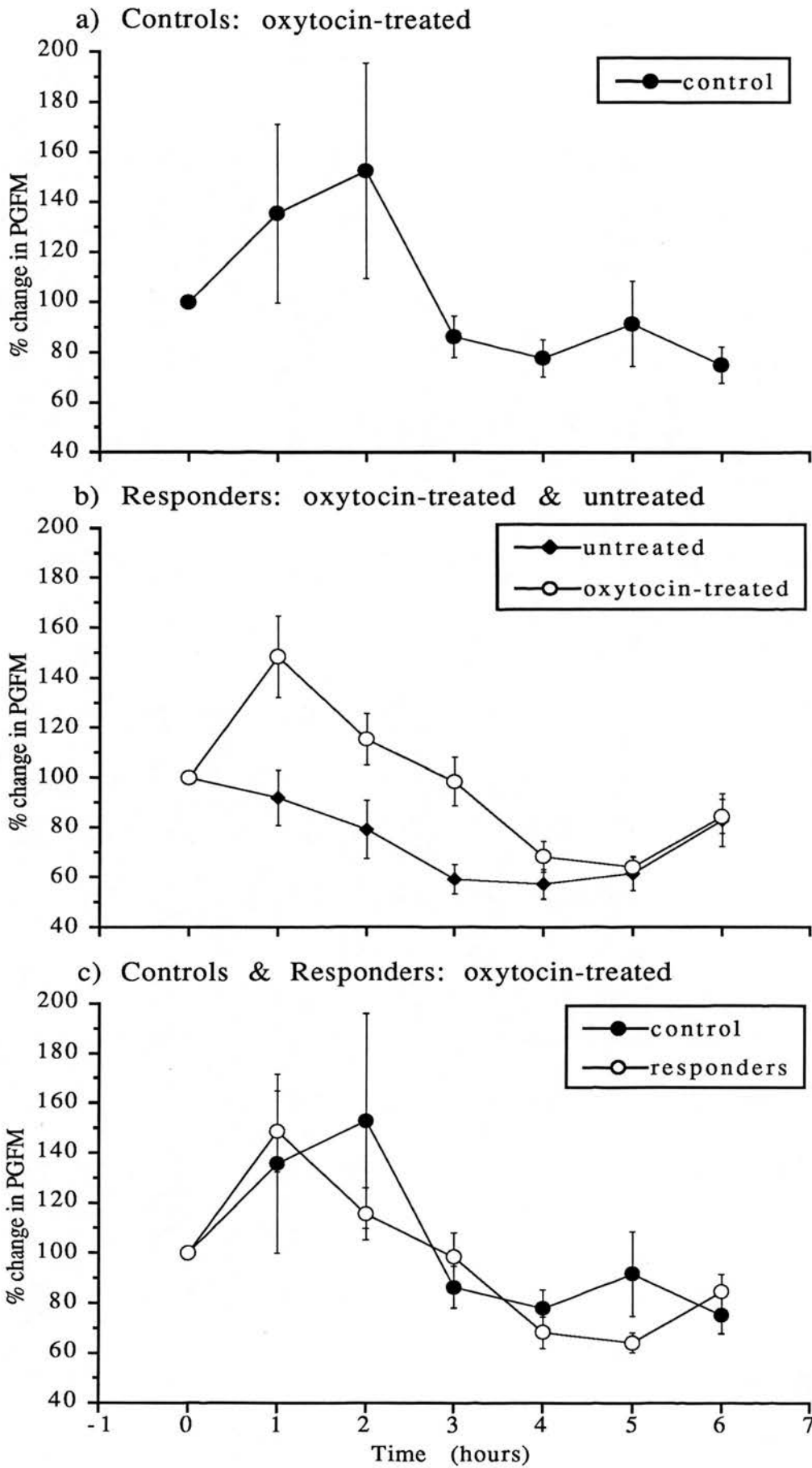


Figure 6.3. Time (post-oxytocin challenge) to maximum PGFM response for the controls (Group A) and the GnRH responders (Groups B1 and B2).

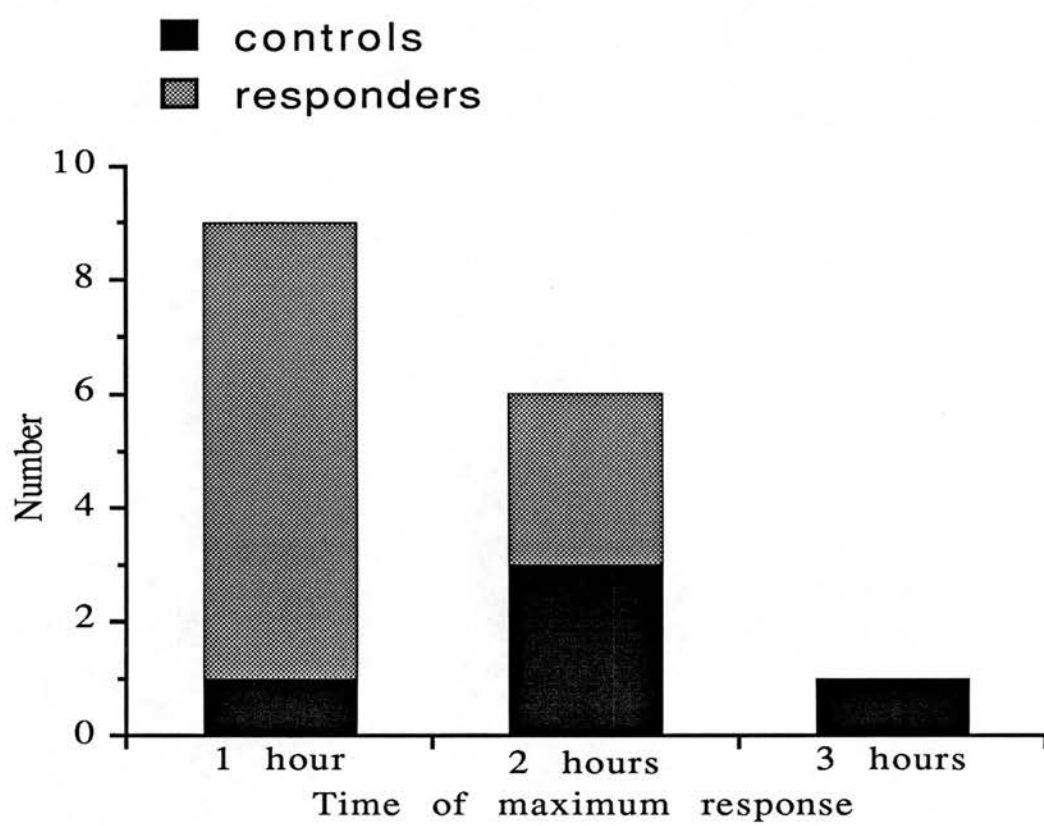
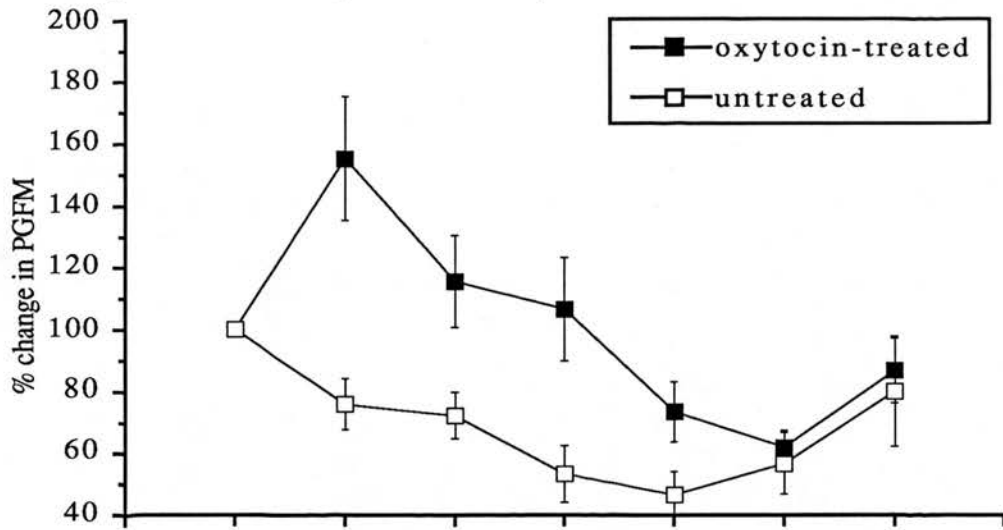
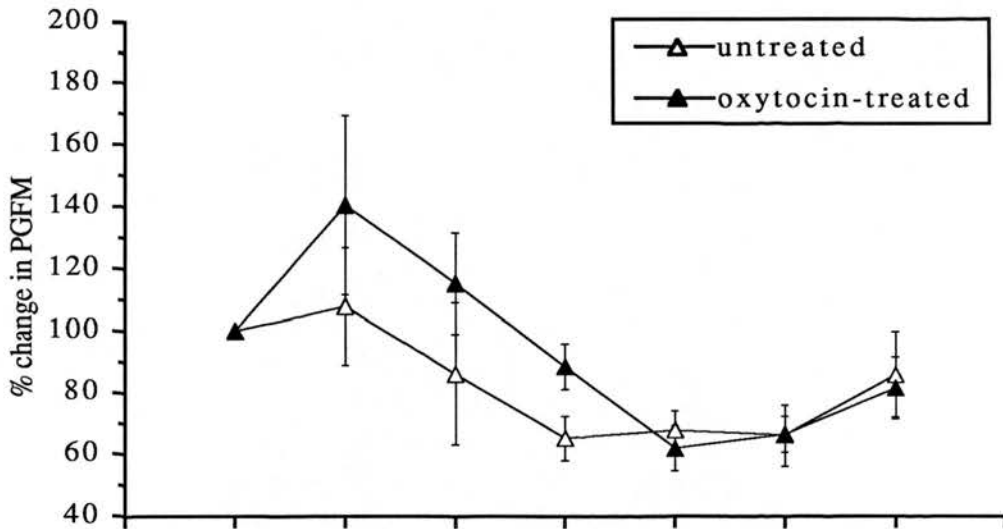


Figure 6.4. Mean (\pm sem) PGFM profiles in the 6-hour period following an oxytocin challenge, expressed as a percentage of mean PGFM concentration in the 4-hour prechallenge period. a) saline-treated responders (n=12); b) bFF-treated responders (n=10); and c) comparison of oxytocin-treated, saline-treated responders (n=6) and oxytocin-treated, bFF-treated responders (n=5). Time 0 = time of oxytocin injection.

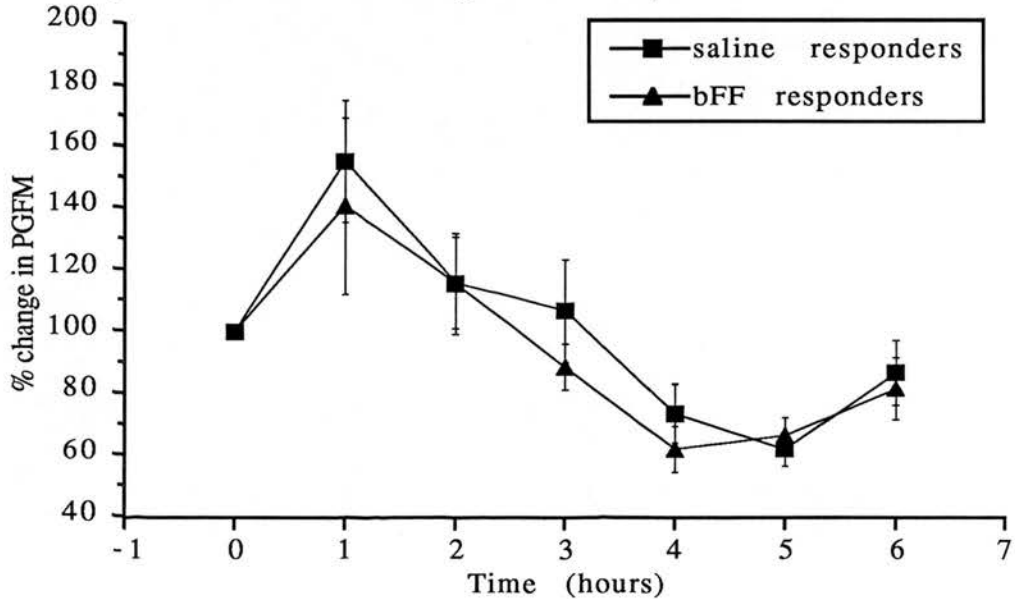
a) Saline Responders: oxytocin-treated & untreated



b) bFF Responders: oxytocin-treated & untreated



c) Saline & bFF Responders: oxytocin-treated



compared in Figure 6.4.c. There was no significant difference in the amount of PGFM secreted between the 2 groups.

The mean (\pm sem) magnitude of the oxytocin-stimulated PGFM response in the saline-treated responders ($60.0 \pm 16.6\%$) was not significantly different from the bFF-treated responders ($56.3 \pm 22.5\%$).

(v) *Pattern of PGFM secretion*

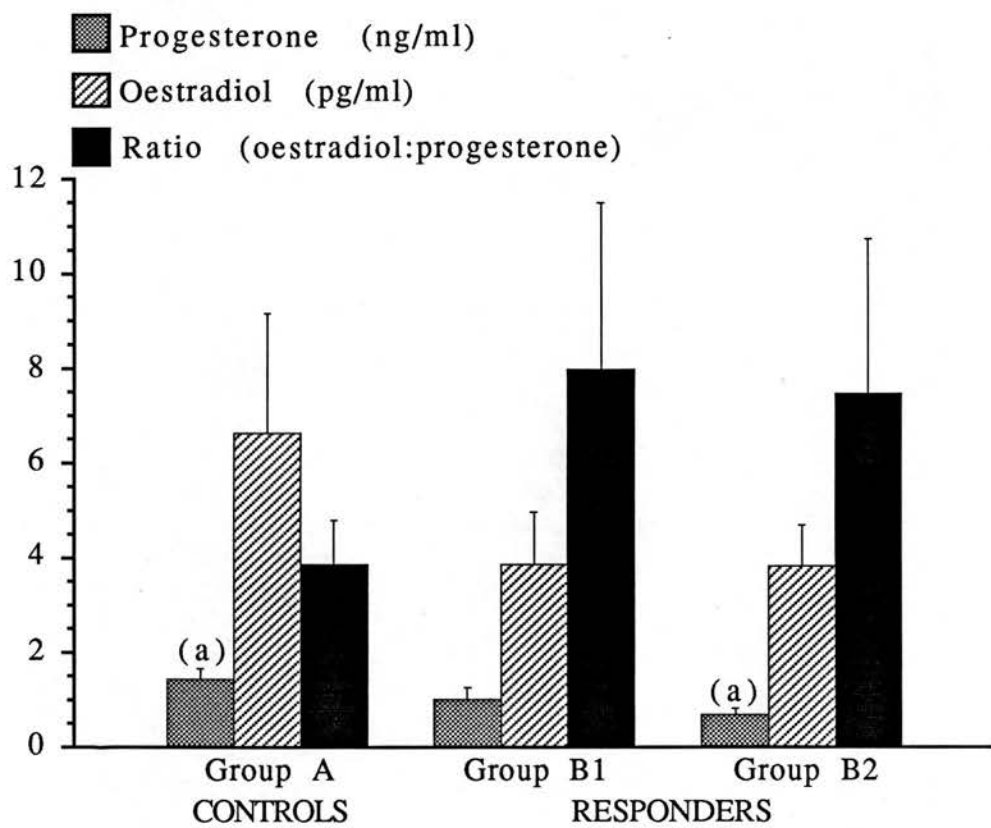
In all 3 groups, mean PGFM concentrations fell below baseline concentrations over the course of the 6-hour post-injection period, before increasing towards pre-injection concentrations. This decline was seen in both oxytocin-treated heifers following the initial oxytocin-induced increase in PGFM, and in untreated heifers. The reason for this pattern of PGFM secretion is not known.

(vi) *Pretreatment steroid concentrations*

Mean (\pm sem) pretreatment concentrations of progesterone and oestradiol and the ratio of oestradiol:progesterone, for the 3 groups are shown in Figure 6.5. Pretreatment progesterone concentrations tended ^($P > 0.05$) to be lower in the responders compared to controls, and this difference was significant ($P < 0.05$) when controls were compared to bFF-treated responders. Pretreatment oestradiol concentrations tended to be higher in controls, whereas the ratio of oestradiol:progesterone tended to be higher in responders when compared to controls.

There was no significant relationship between the PGFM response and pretreatment progesterone concentration, ratio of pretreatment oestradiol to pretreatment oestradiol, total progesterone secreted during the 7-day period preceding the oxytocin challenge, or total progesterone secreted during the

Figure 6.5. Mean (\pm sem) pretreatment progesterone concentration, pretreatment oestradiol concentration and oestradiol:progesterone ratio for controls (Group A: n=5), saline-treated responders (Group B1: n=12) and bFF-treated responders (Group B2: n=10). Means with similar superscripts are different ($P < 0.05$).



4-day interval between Day 3 after ovulation (when the responders received PG3) and the injection of oxytocin.

(vii) *Peripheral progesterone concentrations and temporal characteristics of the oestrous cycle*

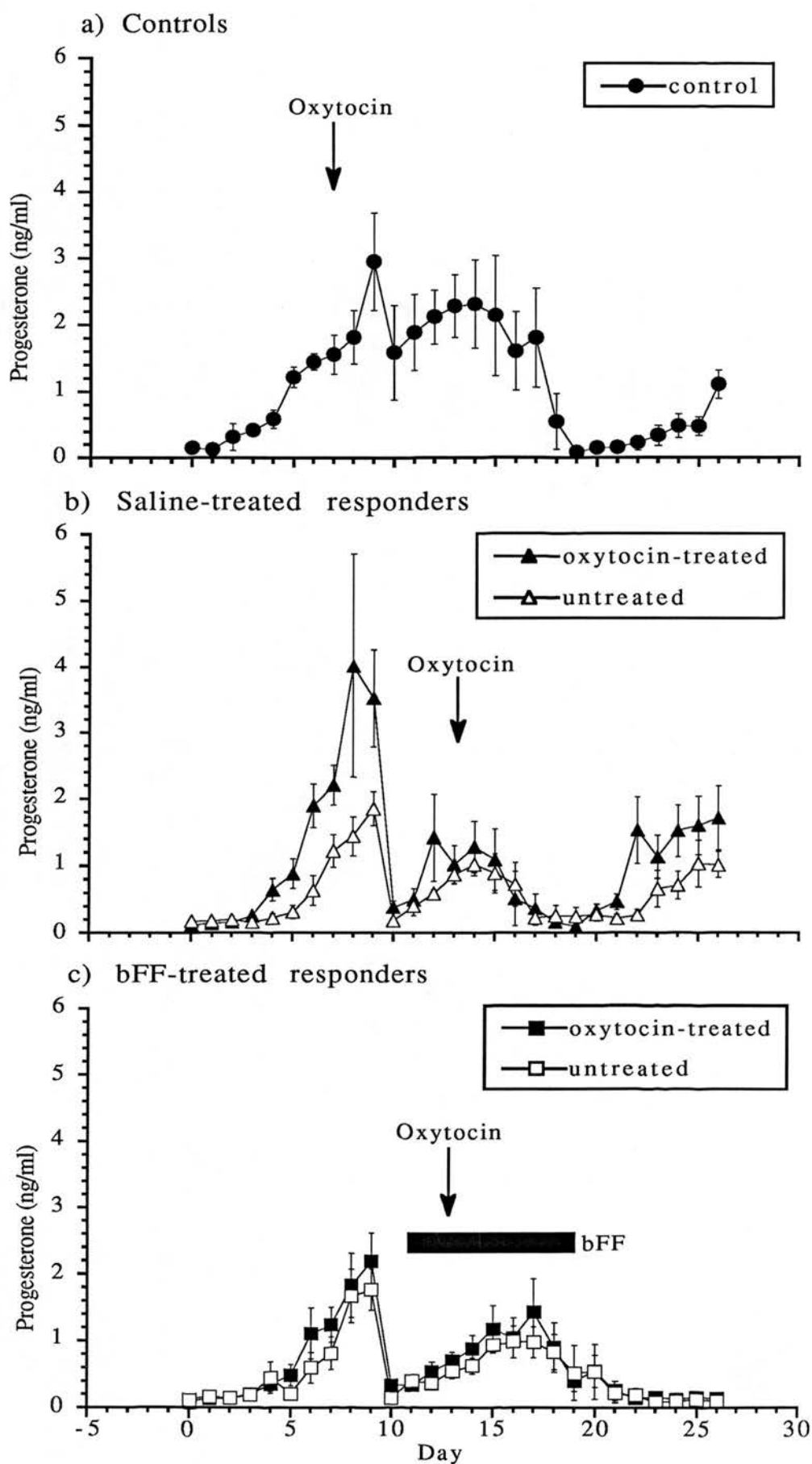
Mean serum progesterone profiles for controls, saline-treated responders and bFF-treated responders are shown in Figure 6.6. Mean oestrous cycle length for controls was 19.8 ± 0.6 days. Mean intervals from GnRH to oestrus for oxytocin-treated and untreated, saline-treated responders were not significantly different (11.5 ± 0.5 days and 11.2 ± 0.3 days respectively). Mean intervals from GnRH to oestrus for the oxytocin-treated and untreated, bFF-treated responders were also not significantly different (18.0 ± 0.4 days and 18.5 ± 0.3 days respectively). In addition, progesterone profiles for saline-treated and bFF-treated animals in the period between the oxytocin challenge and the subsequent oestrus were not different when oxytocin-treated animals were compared to untreated animals.

6.4. Discussion

In discussing this study, peripheral measurements of PGFM are assumed to be an accurate reflection of uterine $\text{PGF}_{2\alpha}$ secretion. The validity of this assumption remains a point of controversy (Silvia *et al.*, 1991).

This study has shown that an oxytocin challenge on Day 7 of the oestrous cycle resulted in a $\text{PGF}_{2\alpha}$ response (measured as PGFM in the peripheral circulation) in heifers with a 7-day old spontaneously-formed CL and in heifers with a 7-day old induced CL. PGFM responses of similar magnitude were observed after oxytocin injection on Day 7 (Lafrance & Goff, 1988), but exceeded those observed on Day 10-11 in the study of Silvia &

Figure 6.6. Mean (\pm sem) serum progesterone profiles for a) controls (n=5); b) saline-treated responders (n=12); and c) bFF-treated responders (n=10). Day 0 = the day of synchronized oestrus. GnRH was given on Day 6 and PGF₂ α (PG3) was given on Day 9. The solid bar indicates the period of bFF treatment, and arrows indicate the day of oxytocin challenge.



Taylor (1989). The magnitude of the PGFM response increased dramatically on Day 14 (Lafrance & Goff, 1988) and in the late (Day 15-17) luteal phase (Silvia & Taylor, 1989), but due to the limited lifespan of the induced CL we were unable to challenge with oxytocin at this stage of the oestrous cycle. Progesterone concentrations started to decline after Day 7 following GnRH administration in the GnRH responders (see Chapter 3, Figure 3.4.).

We have previously shown that the lifespan of the GnRH-induced CL is limited when compared to the endogenous, spontaneously-formed CL (see Chapter 3). Short luteal phases also occur in cattle and sheep following first spontaneous and gonadotrophin-induced ovulations post-partum (Lauderdale, 1986; Hunter, 1991). Garverick *et al.* (1992) concluded that the premature demise of these post-partum CL in cattle was a result of advanced release of $\text{PGF}_{2\alpha}$ from the uterus. High oxytocin receptor concentration (5-fold higher in animals induced to ovulate 21 days post-partum compared to 35-day post-partum and control groups: Wallace, Morgan, Helliwell, Aitken, Cheyne & Williams, 1991) and enhanced $\text{PGF}_{2\alpha}$ release (Wallace, Thompson, Aitken & Cheyne, 1993) are thought to contribute to abnormal luteal function in post-partum ewes. In this study an increase in the PGFM response was not seen in the responders when compared to the control group. Thus, it would appear that the premature demise of the induced CL is not a consequence of advanced release of $\text{PGF}_{2\alpha}$.

Within the limits of the experimental hourly sampling regime, the time of maximum response was significantly advanced in responders compared to the control animals. It is not known why there is a difference in the time course of the PGFM response between the two groups.

The effects of the steroids, progesterone and oestradiol, on uterine secretion of $\text{PGF}_{2\alpha}$ in ruminant species are complex. No significant

differences were observed in the magnitude of the PGFM response between saline-treated responders and bFF-treated responders (in which peripheral oestradiol concentrations are reduced by bFF administration; see Chapter 4). However, animals were challenged with oxytocin 2 days after the commencement of bFF administration when mean oestradiol concentrations and mean ratios of oestradiol to progesterone for saline-treated and bFF-treated groups were not significantly different (see Chapter 4, Figure 4.12. and Figure 4.13.b). The magnitude of the PGFM response on Day 7 of the spontaneous or induced cycle was not related to the total serum progesterone measured on the 7 days preceding the oxytocin challenge. However, for the first 3 days of the lifespan of the induced CL serum progesterone concentrations were supplemented by progesterone secretion from the endogenous, spontaneously-formed CL. Lamming *et al.* (1991) concluded that the effects of progesterone are dominant in the control of oxytocin receptor expression which is crucial to the increase in $\text{PGF}_{2\alpha}$ release associated with luteolysis.

In this study, although the induced CL was 7 days old, the uterus had been exposed to progesterone for 13 days (since the spontaneous ovulation). However, we did not observe an increase in PGFM response in the responders. Lafrance & Goff (1988) observed a 2.5-fold increase in plasma PGFM concentrations in oestradiol-treated, ovariectomized heifers primed with progesterone for 14 days, compared to a 1.4-fold increase after 7 days progesterone priming. On Day 3 after the induced ovulation, administration of exogenous $\text{PGF}_{2\alpha}$ to the GnRH responders resulted in a dramatic decline in mean serum progesterone concentrations with the demise of the spontaneously-formed CL (see Chapter 3, Figure 3.4.) similar to the fall in progesterone concentrations observed during normal luteolysis. The

alteration in the ovarian steroid environment may have influenced the subsequent uterine secretory responsiveness to oxytocin.

Changes in uterine secretory responsiveness to oxytocin may reflect availability of arachidonic acid or its precursors for $\text{PGF}_{2\alpha}$ synthesis, activity of PGH synthase, or endometrial concentrations of receptors for oxytocin (Silvia *et al.*, 1991). Concentrations of endometrial oxytocin receptor change during the oestrous cycle reaching a maximum at oestrus and declining during the mid-luteal phase (Schams *et al.*, 1987). Both stimulatory and inhibitory effects of progesterone on endometrial concentrations of oxytocin receptors have been reported. Endometrial concentration of oxytocin receptors in ovariectomized ewes can be increased by treatment with progesterone for 10 days (Vallet *et al.*, 1990). However, the rise in uterine oxytocin receptors at oestrus is closely linked to declining progesterone concentrations (McCracken, 1980; Sheldrick & Flint, 1985; Fuchs *et al.*, 1990), and elevated concentrations of progesterone prevented the formation of receptors for oxytocin (Soloff, 1975). In addition, although exposure of the uterus to progesterone appears to be necessary for the development of the oxytocin-induced PGFM response, progesterone may also exert an inhibitory effect on uterine $\text{PGF}_{2\alpha}$ secretion. Uterine secretion of prostaglandin increases acutely immediately following lutectomy or ovariectomy in both cattle and sheep (Kindahl *et al.*, 1981; Ottobre *et al.*, 1984; Vincent & Inskeep, 1986). Thus, although in this study the PG3-induced fall in progesterone concentration may stimulate formation of uterine oxytocin receptors, this was not reflected by an increase in uterine secretory responsiveness on the day of oxytocin challenge. This may be a result of progesterone secretion by the induced CL in the interval between PG3 and the oxytocin challenge.

The absence of a significant relationship between the ratio of pretreatment oestradiol:pretreatment progesterone and the PGFM response was similar to the observations of other workers in the middle of the luteal phase (Day 10-11; Silvia & Taylor, 1989). Lafrance & Goff (1988) found a significant synergistic effect of oestradiol with progesterone on oxytocin-induced $\text{PGF}_{2\alpha}$ release only after the uterus had been primed with progesterone for more than 7 days. In this study the uterus of the GnRH responders had been exposed to progesterone since the spontaneous ovulation (13 days). However, as referred to above, the decline in peripheral progesterone concentrations following administration of $\text{PGF}_{2\alpha}$ (PG3) may have influenced the development of synergy between the 2 steroids.

No effect of oxytocin administration on oestrous cycle duration was observed in the controls, the length of the oestrous cycle being approximately 20 days as expected for heifers (Hafez & Sugie, 1963; Robinson, 1977; Robinson & Shelton, 1991). Additionally no effects on oestrous cycle duration or progesterone secretion were observed in the oxytocin-treated responders. In cows and heifers, injections of oxytocin in the early luteal phase inhibited CL development and shortened the oestrous cycle (Black & Duby, 1965; Armstrong & Hansel, 1959; Hansel & Wagner, 1960; Wilks & Hansel, 1971; Newcomb *et al.*, 1971; Milvae & Hansel, 1980b). Luteal function was impaired by oxytocin even if the dose and duration of treatment were insufficient to cause complete inhibition of the CL (Milvae & Hansel, 1980b). Oxytocin treatment during the early luteal phase depressed the ability of the CL to produce the potent luteotrophin PGI_2 (Hansel & Dowd, 1986). These effects occurred only when oxytocin was administered during a critical period (defined as Days 2-6) early in the oestrous cycle, and the presence of the uterus was required for its manifestation. Thus the subsequent

premature demise of the GnRH-induced CL does not appear to be due to a mechanism involving oxytocin-induced reduction in luteotrophic support.

In conclusion, this study has demonstrated that progesterone production by the induced CL is adequate to prime the uterus to respond to an exogenous oxytocin challenge with the release of $\text{PGF}_{2\alpha}$ of similar magnitude to that observed in control animals with a spontaneously-formed CL of the same age. Furthermore, the premature demise of the CL induced by GnRH on Day 6 of the oestrous cycle does not appear to be the result of the premature release of a luteolytic pattern of $\text{PGF}_{2\alpha}$ secretion.

CHAPTER 7

DEVELOPMENT OF A BOVINE LUTEAL CELL CULTURE SYSTEM AND STUDY OF THE EFFECT OF BOVINE FOLLICULAR FLUID FRACTIONS ON PROGESTERONE PRODUCTION BY CULTURED BOVINE LUTEAL CELLS *IN VITRO*

7.1.Introduction

Culture techniques offer a potent tool for the study of effects of hormones on maintenance and regression in the CL. Short-term incubations of luteal tissue slices or dissociated luteal cells exclude the influence and interference of endogenous hormones which are present *in vivo*, but cannot be used to evaluate the long-term effects of hormones on luteal function.

The aim of the first part of this study was to develop a simple, reproducible system for the dispersion and culture of bovine luteal cells, and to evaluate the use of centrifugal elutriation for the separation of large and small steroidogenic cells.

There is increasing evidence that luteal function could be controlled not only by secretions from the adenohipophysis but also by factors secreted within the ovary itself (Williams & Behrman, 1983; Khan-Dawood & Dawood, 1986; Tsafiriri, 1988). The composition and biological effects of

follicular fluid has warranted considerable investigation in recent years. Follicular fluid is composed partly of secretions from the follicle and partly of exudates from plasma. Its composition reflects changes in the secretory processes of the granulosa layer and theca interna, and alterations in the components of the plasma due to physiological or pathological processes.

Intravenous administration of bFF to heifers has been shown to influence the lifespan of the GnRH-induced CL (see Chapter 4). The aim of the second part of this study was to examine the effect of steroid-stripped bFF on progesterone production by cultured bovine luteal cells *in vitro*. We have used red sepharose chromatography followed by anion exchange chromatography to generate inhibin-depleted (R1: unbound to sepharose) and inhibin-enriched (R2: bound to sepharose) fractions of bFF (see Chapter 2) subsequently shown to have stimulatory or inhibitory effects on growth of ovine granulosa cells from small and large follicles in culture R. Webb, (unpublished observations), as assessed by tritiated thymidine uptake (Gong *et al.*, 1992). Fractions previously shown to have maximal stimulatory or inhibitory effect on granulosa cells were selected to investigate their effect on cultured luteal cells.

7.2. Materials and Methods

7.2.1. Experimental procedure for luteal cell dispersion

Bovine ovaries were collected from a local abattoir into Medium 199 (Gibco Life Technologies Ltd., Paisley, Scotland) containing antibiotics and sodium bicarbonate (holding medium) at 37°C in a thermos-flask and transported to the laboratory within 1 hour of slaughter. The CL were decapsulated and weighed and divided into 1 g pieces. Each piece was weighed and minced in holding medium. Dispersion of bovine luteal cells was carried out as detailed in Chapter 2 (see section 2.8.) following

preliminary studies to optimise experimental conditions as described below. Unless otherwise stated all reagents were from Sigma Chemical Co. (Poole, Dorset, UK).

7.2.1.1. Preliminary study to compare 4 collagenase types

Four enzyme solutions (see Table 7.1.; A-D) were prepared. Medium 199 containing antibiotics and sodium bicarbonate (5 ml/g luteal tissue) was supplemented with 20mM Hepes, 1% BSA, collagenase (2000 U/g luteal tissue; solution A-type I collagenase, solution B-type II collagenase, solution C-type V collagenase, solution D-type XI collagenase) and DNase (1 mg/g luteal tissue).

Luteal tissue was collected and processed as described above, and bovine luteal cells were obtained by collagenase dispersion, as described in Chapter 2 (see section 2.8.). Each CL was incubated with each of the 4 enzyme solutions. The experiment was performed with 5 CL. Cell yield is shown in Table 7.1.

Table 7.1. Mean (\pm sem) cell yield following dispersion with 4 different collagenase types.

SOLUTION	COLLAGENASE TYPE	CELL YIELD/ML: MEAN (\pm SEM) X 10^4
A	I	9.4 \pm 1.4
B	II	14.4 \pm 3.1
C	V	21.1 \pm 1.6
D	XI	3.8 \pm 1.5

Cell yield with type V collagenase was significantly greater than cell yield with type I and type XI collagenase ($P < 0.001$). As a result of this

preliminary study, type V collagenase was used in all subsequent luteal cell dispersions.

7.2.1.2. Preliminary study to compare incubation media with and without Ca^{2+} and Mg^{2+}

Two enzyme solutions (A and B) were prepared. Solution A contained Medium 199 (5 ml/g luteal tissue) containing antibiotics and sodium bicarbonate, supplemented with 20mM Hepes, 1% BSA, type V collagenase (2000 U/g luteal tissue) and DNase (1 mg/g luteal tissue). Solution B contained Medium 199 (5 ml/g luteal tissue) containing antibiotics, Earle's salts and Hepes, supplemented with 1% BSA, type V collagenase (2000 U/g luteal tissue) and DNase (1 mg/g luteal tissue).

Luteal tissue was collected and processed as described above, and bovine luteal cells were obtained by collagenase dispersion, as described in Chapter 2 (see section 2.8.). Each CL was incubated with each of the 2 enzyme solutions. The experiment was performed with 3 CL. Cell yield is shown in Table 7.2.

Table 7.2. Mean (\pm sem) cell yield following dispersion with 2 different incubation media.

SOLUTION	MEDIUM	CELL YIELD/ML: MEAN (\pm SEM) $\times 10^4$
A	M199	49.6 \pm 3.7
B	M199+EARLES SALTS	35.5 \pm 5.5

As a result of this preliminary study Medium 199 without Ca^{2+} and Mg^{2+} was used in all subsequent luteal cell dispersions.

7.2.2. Experimental procedure for luteal cell separation

Separation of dispersed bovine luteal cells into large and small cell fractions was carried out on an elutriator as detailed in Chapter 2 (see section 2.9.) following a preliminary study to optimise experimental conditions, as described below.

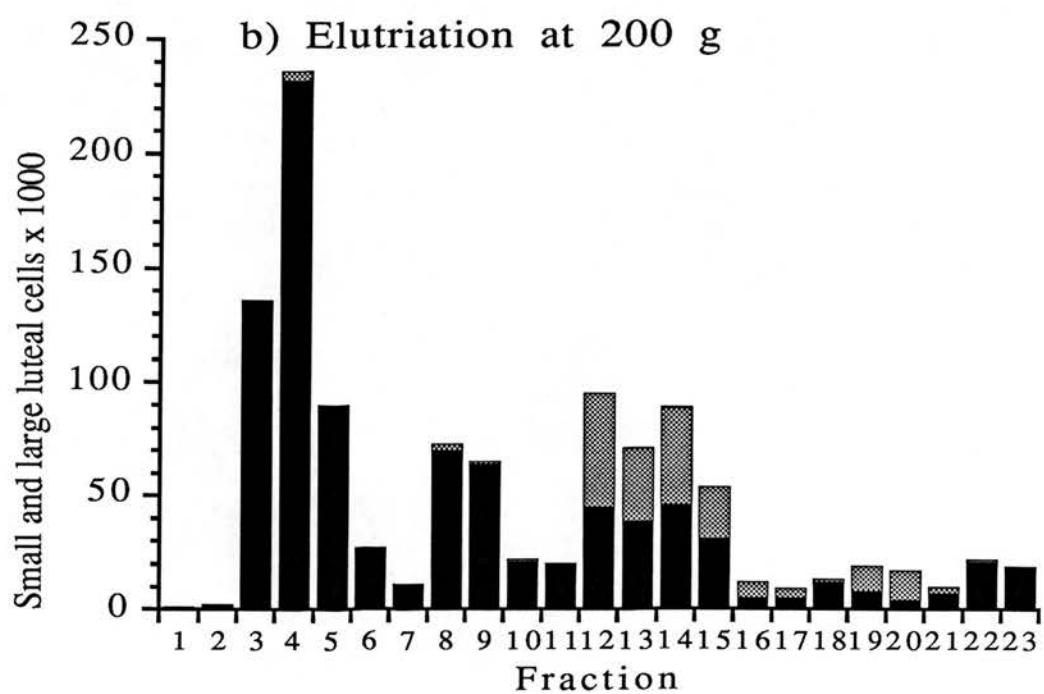
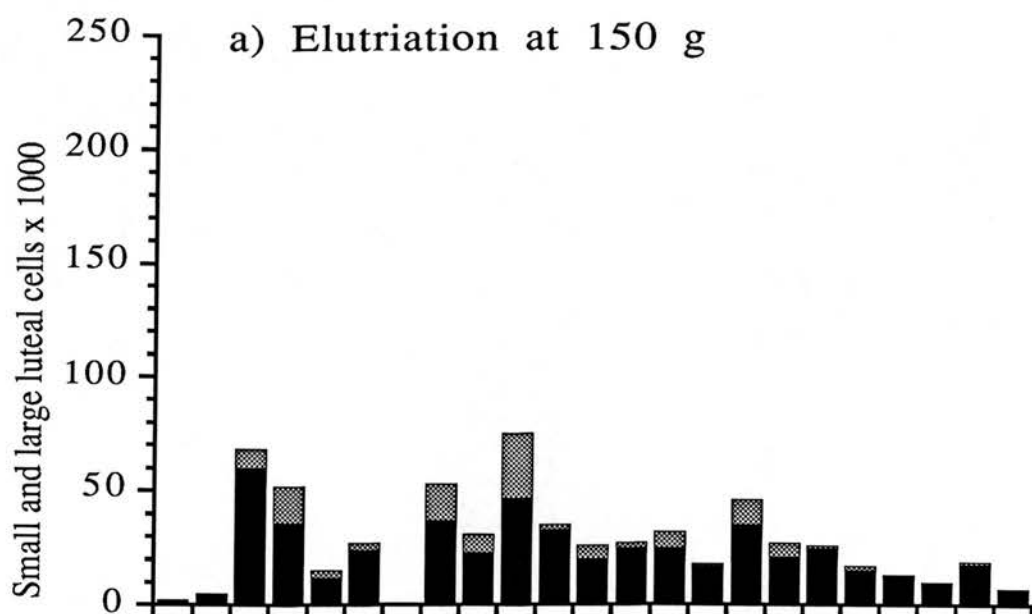
7.2.2.1. Preliminary study to compare 2 elutriation rates

Dispersed luteal cells were suspended in elutriating buffer (Chapter 2, section 2.9.) at a known concentration. Two 5 ml aliquots were taken. The first aliquot was elutriated at 150 g, and the second aliquot was elutriated at 200 g. Fractions (23 x 10 ml) were obtained from each aliquot post-elutriation. Following centrifugation at 100 g for 5 minutes, each cell pellet was resuspended in 0.5-2 ml post-elutriation solution, and cell number/fraction estimated using a haemocytometer. This allowed calculation of total numbers of elutriated cells. Luteal cells were classified as small cells ($\geq 10 \mu\text{m}$ and $< 25 \mu\text{m}$ diameter) or large cells ($\geq 25 \mu\text{m}$ diameter) (Koos & Hansel, 1981). The numbers of cells/fraction are shown in Figure 7.1. Cell recovery from the elutriator is shown in Table 7.3.

Table 7.3. Cell recovery following separation at 2 different elutriation speeds.

TOTAL CELLS x 10 ⁶ PRE- ELUTRIATION	ELUTRIATION SPEED g	TOTAL CELLS x 10 ⁶ POST- ELUTRIATION	% RECOVERY
3.32	150	0.63	19.0
3.32	200	1.13	33.5

Figure 7.1. Numbers of cells/fraction following separation of a luteal cell suspension on an elutriator at a speed of 150 g (upper panel) and 200 g (lower panel). The black columns represent small luteal cells and the grey columns represent large luteal cells.



As a result of this preliminary study all subsequent cell separations on the elutriator were carried out at 200 g.

7.2.3. Experimental procedure for luteal cell culture

7.2.3.1. Separated populations of small & large luteal cells

Collection of bovine luteal tissue, and dispersion and separation of luteal cells was carried out as described above. Following elutriation, fractions 3-5 inclusive were combined to generate a suspension of predominantly small luteal cells, and fractions 15-20 inclusive were combined to generate a suspension enriched with large luteal cells. An aliquot was removed from each fraction for assessment of cell number and viability. Following centrifugation at 100 g for 5 minutes and removal of the supernatant, each fraction was resuspended in culture medium (Medium 199 containing antibiotics, Earle's salts and Hepes), to achieve a final concentration of 0.5×10^6 cells/ml.

Two 48-well culture plates were prepared as described in Chapter 2 (see section 2.10.). The culture medium was poured off the wells. Aliquots (250 μ l) of small luteal cell suspension were dispensed to the 24 central wells of plate 1 (125,000 small luteal cells/well), and aliquots (250 μ l) of large luteal cell suspension were dispensed to the 24 central wells of plate 2 (125,000 large luteal cells/well). The plates were incubated overnight at 37°C in an atmosphere of 2% carbon dioxide and 98% air.

Following examination of the plates to assess the condition of the cells, medium was removed from each well and stored at -40°C for future progesterone assay. If no further treatment was carried out, culture plates were stored at -40°C. Cell density/well was determined by DNA assay.

7.2.3.2. Mixed populations of small & large luteal cells

The procedure was as described above, but omitting cell separation in the elutriator. Mixed luteal cell suspension (250 μ l) was dispensed to the 24 central wells of plates 1 and 2 (125,000 luteal cells/well).

7.2.4. Experimental procedure for bFF fraction challenge

After 24 hours plating-down period, following the procedure outlined above for cell dispersion and culture of mixed luteal cells, the cells were challenged with the following treatments. Each treatment was performed in triplicate on 2 plates.

- (1) Response to LH: The dose used in this study was 1 μ g/ml (final concentration in each well) bovine LH (USDA bLH-B-5, USDA-Reproduction Lab., Beltsville, MD 20705). Freeze-dried bovine LH was suspended in PBS to achieve a concentration of 25 μ g/ml (Solution A). ?
- (2) Response to bFF fractions: Three bFF fractions were prepared in our laboratory as described in Chapter 2 (see section 2.7.2.). Three bFF fractions with previously demonstrated effects in granulosa cell cultures: R2₁ (bound to red sepharose) and R1₁ and R1₂ (both unbound to red sepharose) were diluted 1:450 (equivalent to 20 μ g protein/ml), 1:450 (equivalent to 20 μ g protein/ml) and 1:2000 (equivalent to 40 μ g protein/ml) respectively in culture medium (Solutions F1, F2 and F3). The dilution of each bFF fraction from stock was carried out twice, with each dilution being used on separate plates to ensure no dilution error. However, there was no difference in response and results from the 2 plates were pooled.

Treatments included: control (250 μ l culture medium); LH alone (10 μ l Solution A plus 250 μ l culture medium); F1 alone (250 μ l Solution F1); F1 plus LH (250 μ l Solution F1 plus 10 μ l Solution A); F2 alone (250 μ l Solution F2); F2 plus LH (250 μ l Solution F2 plus 10 μ l Solution A); F3 alone (250 μ l Solution F3); F3 plus LH (250 μ l Solution F3 plus 10 μ l Solution A).

After 4 hours, medium was removed from each well and stored at -40°C for future progesterone assay. Culture plates were stored at -40°C , and cell density/well was determined by DNA assay.

7.2.5. Progesterone assay

Media samples from these studies were assayed for progesterone in one assay. The sensitivity of the assay was 13.9 pg/tube. The intra-assay coefficient of variation was 8.4%.

7.2.6. DNA assay

Cultured luteal cells from these studies were assayed for DNA in 2 assays.

7.2.7. Statistical analysis

All data is presented as means \pm sem. Differences between treatments were compared using ANOVA.

In the discussion, when the possible significance of the effects of bFF fractions on progesterone production *in vitro* is considered, total progesterone production *in vivo* by the saline-treated responders and the 8-day bFF-treated responders in Experiments 2 and 3 (Chapter 4) was compared between Day 11 and 14 (Student's *t* test). Differences between daily mean serum oestradiol concentrations for the saline-treated and bFF-treated

responders in Experiment 3 were tested by fitting quadratic curves to the oestradiol profiles for the 2 groups during Days 13-17 (Days 7-11 after GnRH) and comparing the fitted values.

7.3.Results

7.3.1. Development of a bovine luteal cell culture system

The results of cell DNA and basal progesterone production by dispersed bovine small and large luteal cells after 24 hours in culture are shown in Table 7.3. Cell number was calculated from DNA content (assuming 5 pg DNA/cell: Spector, 1956).

Table 7.3. Basal progesterone production by small and large luteal cells during 24 hours in culture.

	SMALL LUTEAL CELLS	LARGE LUTEAL CELLS
MEAN (± SEM) FLUORESCENCE/WELL	2.4 ± 0.1	3.0 ± 0.1
DNA (NG/WELL)	207	250
CELLS X 10 ⁵ /WELL	0.4	0.5
MEAN (± SEM) PROGESTERONE PRODUCTION (NG/ML)	14.5 ± 0.4	13.8 ± 0.7
BASAL PROGESTERONE PRODUCTION (NG/10 ⁵ CELLS/24 HOURS)	9.1	6.9

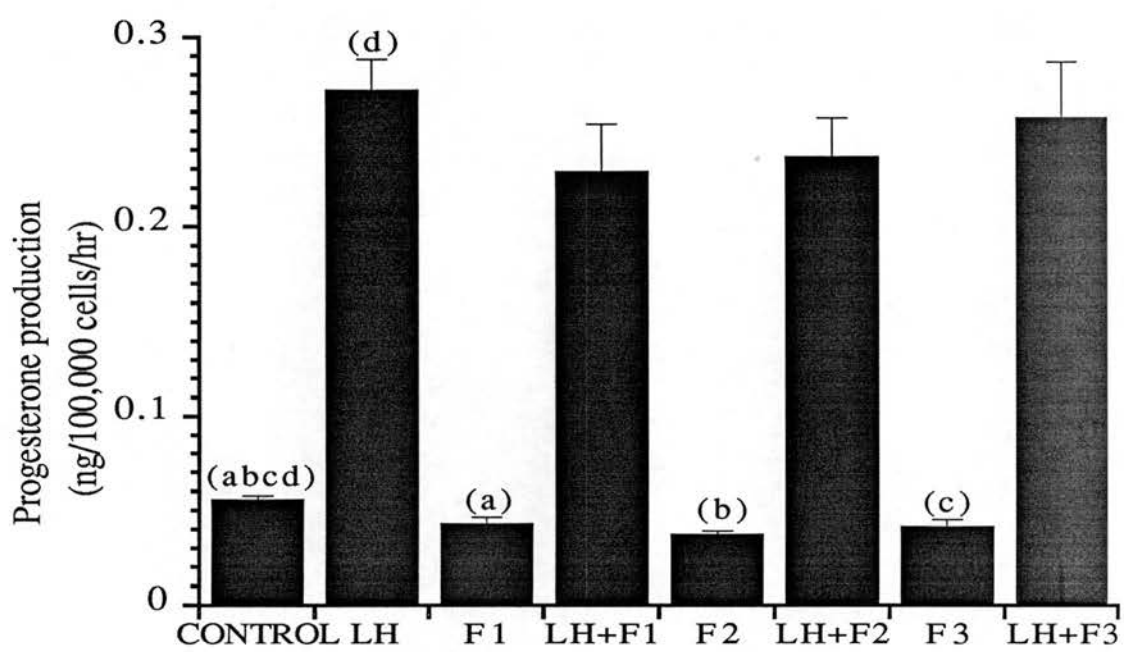
7.3.2. *In vitro* study of the effect of bFF fractions on progesterone production by cultured bovine luteal cells

Mean progesterone production in the 4-hour challenge period is shown in Figure 7.2. LH significantly increased progesterone production by cultured luteal cells when compared to controls ($P<0.001$). All 3 bFF fractions significantly inhibited progesterone production when compared to controls ($P<0.001$). However, addition of LH to the medium overcame the inhibitory effects of all three fractions. There was a slight reduction in the magnitude of the stimulatory response of LH to progesterone production in the presence of the bFF fractions, but this did not reach statistical significance.

7.4. Discussion

A procedure for the dispersion and culture of bovine luteal cells has been developed. The enzymatic dispersion of luteal cells is based on the methods described by Simmons *et al.* (1976) and Harrison *et al.* (1987). The most effective collagenase was type V, a crude preparation from *Clostridium histolyticum* which contains neutral protease and clostripain (a trypsin-like enzyme). A variety of enzymes have been utilized by other workers to obtain cells from luteal tissues including crude and purified trypsin, subtilisin, hyaluronidase, and crude and purified collagenase. The best results were obtained with crude collagenase, which was twice as effective as its highly purified form with respect to dissociation time (Gospodarowicz & Gospodarowicz, 1972, 1975) indicating that other proteolytic enzymes in the preparation were beneficial. DNase was incorporated in the dissociation medium to inhibit cell reaggregation as a result of DNA release. The inclusion of EDTA to prevent cell clumping was contraindicated as it affected cell viability and inhibited collagenase activity. Cell yield was greater in incubation medium without Ca^{2+} and Mg^{2+} (although collagenase requires

Figure 7.2. Mean (\pm sem) progesterone production (n=6 replicates per treatment) by cultured bovine luteal cells (ng progesterone/ 10^5 cells/hour) in the 4-hour treatment period after challenge with LH, bFF fraction 1 (F1) \pm LH, bFF fraction 2 (F2) \pm LH, or bFF fraction 3 (F3) \pm LH. Means with similar superscripts are different ($P < 0.001$).



Ca^{2+} for activity and DNase has a specific requirement for Mg^{2+}). Dispersions were therefore carried out in Ca^{2+} - Mg^{2+} free Medium 199. Gospodarowicz & Gospodarowicz (1972) found that Krebs Ringer bicarbonate buffer plus glucose (KRBG) failed to maintain bovine luteal cells through the dissociation and incubation procedures, but fortification of KRBG with 4% BSA produced satisfactory results. There are disadvantages to the use of BSA, since it contains a small but variable amount of LH. Furthermore, BSA (and other common cell culture additives such as fetal calf serum) protect cells from stains such as trypan blue, and result in erroneously high viability ratings. Thus, cells rendered metabolically inert by heat treatment produced excellent viability ratings in the presence of serum (Tennant, 1964). For these reasons, chemically-defined media were used throughout and BSA was included at the lower concentration of 1%.

Hoyer, Kong, Crichton, Bevan & Krutzsch (1988) observed changes to the typical steroidogenic ultrastructure of the bovine luteal cell as a function of dissociation. There were more lipid droplets and rough endoplasmic reticulum and a virtual absence of secretory granules in cells examined from Day 10 dissociated tissue preparations. Loss of secretory granules during the dissociation procedure has also been reported by Rodgers & O'Shea (1982).

Pretreatment of culture plates with donor calf serum promoted cell attachment. The serum-containing medium was removed by washing the plates with three volumes of Medium 199. The luteal cells did not appear to proliferate and did not form a monolayer in the serum-free culture conditions used, but flattened and elongated, forming small aggregates. Similar observations of bovine luteal cells in culture were made by McArdle & Holtorf (1989). However these workers found that pretreatment of the plates with substrates to aid attachment was not necessary.

In our studies cells were cultured in serum-free medium and responded to LH after 24 hours with a dramatic increase in progesterone production. Although the ability of bovine luteal tissue to secrete progesterone was increased in the presence of small amounts of serum (Condon & Pate, 1981), addition of LH to cells cultured in serum-containing medium did not increase progesterone production (Pate & Condon, 1982). However, when the luteal cells were cultured in serum-free medium, LH produced a dose-dependent increase in progesterone production during the first 24 hours of culture. Responsiveness was lost during the period from Day 1-3 but then returned as the culture progressed (Pate & Condon, 1984). Similarly, Kong, Marion & Hoyer (1989) found that progesterone secretion was not stimulated by LH (10 ng/ml or 100 ng/ml) at any time during culture of ovine luteal cells maintained in M199 containing 5% calf serum. Calf serum contains LH and it is possible that cells cultured in serum-containing media are already maximally stimulated and so do not respond to LH with an increase in progesterone production.

Luteal cells were assessed after 24 hours in culture. Plating was observed to be optimal by 24 hours in ovine luteal cell cultures (Hoyer *et al.*, 1988). In our study both separated and mixed cells were plated at a density of 0.5×10^6 cells/ml (i.e. 1.25×10^5 cells/well). In experiments in which cells were plated at variable densities (2.5×10^4 - 2.5×10^5 cells/plate), accumulation of progesterone was decreased with increasing densities of cells on all days of culture (Hoyer *et al.*, 1988). The importance of plating density should be reflected by the use of different cell concentrations when separated populations of small and large luteal cells are cultured. For example, if the mean cell diameter of small luteal cells is 15 μ m and the mean cell diameter of large luteal cells is 30 μ m, comparable densities would be achieved by plating small luteal cells down at twice the concentration of

large luteal cells. However, in terms of cell areas of $707 \mu\text{m}^2$ for a small luteal cell of $15 \mu\text{m}$ diameter, and $2827 \mu\text{m}^2$ for a large luteal cell of $30 \mu\text{m}$ diameter, comparable densities would be achieved by plating small luteal cells down at four times the concentration of large luteal cells. Optimal plating densities for small and large luteal cells warrant further investigation for future work with separated populations of steroidogenic cells.

Centrifugal elutriation was of limited use as a procedure for generating purified fractions of small and large luteal cells prior to cell culture. There was an unavoidable loss of some cells in the elutriator because a volume of the cell suspension was retained in the sample chamber. The incorporation of BSA into the elutriating buffer to coat the tubing preventing cell adhesion, but increased the viscosity of the buffer making cells 'lighter', with the result that large cells tended to elute earlier. Although relatively pure fractions of small luteal cells were obtained, there was contamination of the large luteal cells with some small cells. This contamination consisted in part of incompletely dissociated small luteal cells as well as small luteal cells attached to large luteal cells. Reducing the speed of the rotor to concentrate the cells further reduced the efficiency of separation as large cells tended to leave the elutriator earlier for a given speed of the peristaltic pump. Furthermore, the process of elutriation was non-sterile and may have compromised subsequent cell viability in culture. Basal progesterone production was low: $9.1 \text{ ng}/10^5$ small luteal cells in 24 hours, compared to $15 \text{ ng}/10^5$ small luteal cells in a 2 hour incubation (El boudrari, Benhaim & Leymarie, 1990). Mean percentage of separated cells which plated down (36%) was low when compared to the percentage of non-elutriated, mixed cells plated down (>85%), and the percentage of bovine luteal cells plated down (>70% attachment) in the study of McArdle & Holtorf

(1989). Cell viability following elutriation was good, but results may have been artificially high due to incorporation of BSA in the elutriating buffer. It is possible that precursors for progesterone synthesis by the luteal cells were limiting (see Chapter 8). On a per cell basis, basal progesterone production was lower in large luteal cells when compared to small luteal cells, possibly due to presence of contaminating small luteal cells in the large cell fraction, to differential effects of elutriation on viability of small and large luteal cells, or to the adoption of suboptimal plating densities. In other studies large luteal cells in sheep (Fitz *et al.*, 1982) and cattle (Koos & Hansel, 1981) secreted 20 times as much progesterone as small luteal cells basally.

The study of the *in vitro* effect of bFF fractions on progesterone production by cultured luteal cells has shown that both inhibin-enriched and inhibin-depleted bFF fractions significantly inhibited progesterone production in a 4-hour period. Addition of LH to the medium overcame the inhibitory effect of all 3 follicular fluid fractions indicating that the follicular fluid fractions did not compromise luteal cell viability. In contrast, only F3 had an inhibitory effect on granulosa cell growth, whilst both F1 and F2 had a stimulatory effect on granulosa cell growth. Effects of follicular fluid fractions on progesterone production in luteal cell cultures and cell growth in granulosa cell cultures, were independent of inhibin content.

Ledwitz-Rigby *et al.* (1977) have shown that follicular fluid from small porcine follicles reduced the accumulation of cAMP in response to LH and decreased the secretion of progesterone from cultured granulosa cells obtained from large follicles. In a subsequent study by Shemesh (1979), addition of charcoal-extracted mid-luteal phase bFF to the culture medium caused a two-fold decrease in the accumulation of both progesterone and $\text{PGF}_{2\alpha}$ by the cultured granulosa cells. In contrast, when preovulatory bFF

was used there was no inhibitory effect on secretion of progesterone and PGF_{2α}. Furthermore, in the presence of 1 µg LH/ml the inhibitory effect of mid-luteal phase bFF on progesterone and PGF_{2α} secretion was abolished. They suggested that bFF from mid-luteal phase follicles inhibited prostaglandin synthetase as well as luteinization. Other reports have mentioned inhibition of granulosa cell secretions *in vitro* by follicular fluid from the human (Hillensjo, Chari, Nilsson, Hamberger, Daume & Sturm, 1983) and sheep (Kadam, Moodbidri & Nandedkar, 1984).

Evidence in sheep *in vivo* indicates a direct (i.e. not mediated by FSH) inhibitory effect of steroid-free bFF on follicular development (Cahill, Clarke, Cummins, Driancourt, Carson & Findlay, 1985a; Cahill, Driancourt, Chalmley & Findlay, 1985b; Fry, Clarke & Cahill, 1987). Although purified inhibin has been shown to have direct effects on ovarian function *in vitro* (Hillier, 1991) and *in vivo* (Woodruff, Lyon, Hansen, Rice & Mather, 1990), it is not clear whether such actions account for the observed effects of crude bFF since several other non-steroidal components of follicular fluid can also act directly on the ovary (see reviews by Tonetta & di Zerega, 1989; Findlay, Xiao & Shukovski, 1990).

A low MW factor (<10,000 Da), likely to be a peptide, which is able to inhibit LH-induced steroidogenesis in small bovine luteal cells *in vitro* markedly, has been demonstrated in bFF by El boudrari *et al.* (1990). This factor suppressed LH-stimulated cAMP accumulation, LH-stimulated progesterone synthesis and dbcAMP induced-progesterone production. Since the inhibition was reversible this factor was unlikely to exert its short-term effects by affecting the viability of the cells. The inhibitory effect was not due to the activation of a proteolytic system by the removal of a protease inhibitor, since preincubation of the fraction with a protease inhibitor did not alter its inhibitory activity. It was unlikely that the observed

antigonadotrophic inhibitory activity was a consequence of bacterial contamination, as reported by other authors (Sluss & Reichert, 1983) for effects of porcine follicular fluid on granulosa progesterone production, since collection of bFF under sterile conditions did not alter its inhibitory property (El boudrari *et al.*, 1990). Since the bFF was treated with charcoal the fraction was unlikely to have been either a nucleotide or a lipid substance such as steroid or prostaglandin. Moreover, the inhibitory activity was not extractable by organic solvents.

The effect of bFF on serum progesterone concentrations *in vivo*, during the initial 4 days of administration, was variable (see Chapter 4). In Experiment 2, during the initial 4 days of bFF administration (Days 5-8 after GnRH injection) (see Figure 4.9.b) mean total progesterone production by the saline-treated responders was not significantly different from mean total progesterone production by the 8-day bFF-treated responders. In contrast, in Experiment 3, mean total progesterone production was reduced in the bFF-treated group on Days 1-4 of bFF administration (see Figure 4.11.) when compared to the saline-treated group ($P < 0.05$). The mean serum oestradiol concentration for the bFF-treated group in Experiment 3 was not significantly reduced when compared to the saline-treated group, until Day 8 after GnRH injection (the 4th day of bFF administration; $P < 0.05$) (see Figure 4.12.). It is possible that the administered bFF may have a direct inhibitory effect on progesterone production by the induced CL, which is apparent in the first 4 days of administration when serum oestradiol concentrations are similar between groups, but serum progesterone concentrations are reduced in the bFF-treated group.

Luteal function was reduced in ewes by bFF in one study (Larson *et al.*, 1987), but not in another (Wallace & McNeilly, 1985). LH-stimulated secretion and (or) synthesis of progesterone by CL *in vitro* was not altered by

treatment with bFF *in vivo* on Days 5 to 11 in the ewe (Larson, Mallory, Dailey & Lewis, 1991), and injections of bFF did not alter plasma progesterone concentrations before or after PGF₂ α -induced luteolysis in heifers in the study of Johnson & Smith (1985).

Follicular fluid contains numerous other non-steroidal, non-inhibin components (Ireland, 1987; Tonetta & di Zerega, 1989). Fully characterized factors known to be present include activin, follistatin, TGF- α and TGF- β (Ying, 1988), EGF (Radford, Panaretto, Avenell & Turnbull, 1987), insulin-like growth factor-binding protein (Shimasaki, Shimonaka, Ui, Inouye, Shibata & Ling, 1990), oxytocin and GnRH-like protein (Wathes *et al.*, 1984; Tsafiriri, 1988). Uncharacterized components include 'ovarian inhibitory protein' and 'follicle regulatory protein' which may act as inhibitors of aromatase activity in granulosa cells (see Tonetta & di Zerega, 1989), 'FSH receptor-binding inhibitor' (Sluss, Fletcher & Reichert, 1983), 'antimitotic activity' (Cahill *et al.*, 1985a; Cahill *et al.*, 1985) and 'gonadotrophin surge-attenuating factor' (Fowler, Messinis & Templeton, 1990; Whitehead, 1990). In addition, CL extracts in many species have been shown to contain a substance referred to as luteinizing hormone receptor-binding inhibitor (LHRBI) which may be present in bFF, and inhibit LH binding to its receptor and so decrease progesterone biosynthesis (Yang, Samaan & Ward, 1976; Kumari, Tucker & Channing, 1979; Nakano, Iwasaki & Yamoto, 1985).

In conclusion we have developed a simple procedure for the dispersion and culture of bovine luteal cells in our laboratory. The process of centrifugal elutriation warrants further refinement to generate fractions of small and large luteal cells of acceptable purity and viability. The results of the second study demonstrate that bFF contains a factor(s) that inhibits progesterone production by mixed populations of cultured luteal cells *in*

vitro. The inhibitory effect is seen in both steroid-stripped, inhibin-enriched fraction and steroid-stripped, inhibin-depleted fractions.

CHAPTER 8

STUDY OF PROGESTERONE PRODUCTION *IN VITRO* BY DISPERSED BOVINE LUTEAL CELLS FROM THE GnRH- INDUCED CORPUS LUTEUM

8.1.Introduction

A simple and highly reproducible procedure for the dissociation of bovine corpora lutea into isolated cell suspensions has been described (see Chapter 2, section 2.8. and Chapter 7). The use of cell suspensions for biological studies offers several advantages: (i) the physical and hormonal environment of the cell can be easily controlled and monitored; (ii) many biochemical parameters which cannot be measured *in vivo* are more easily approached *in vitro*; and (iii) the ability to distribute cell suspensions homogeneously alleviates much of the variability associated with other *in vitro* systems. Furthermore, the use of dispersed cells circumvents difficulties associated with the diffusion of gases, metabolites and substrates in or out of bulk tissues in the form of slices or large fragments and avoids the formation of artifacts associated with tissue trauma or damage due to local anoxia. The time required to generate measurable quantities of products of interest is frequently shorter for dispersed cells than for bulk tissue *in vitro* because of more rapid exchange in the former.

Progesterone synthesis in the bovine CL is stimulated by LH *in vivo* (Simmons & Hansel, 1964; Donaldson *et al.*, 1965; Brunner *et al.*, 1969; Carlson, Norimoto & Hansel, 1971) and *in vitro* (Mason, Marsh & Savard, 1962; Mason & Savard, 1964; Seifert & Hansel, 1965; Armstrong & Black, 1966). The binding of LH to specific luteal cell membrane receptors activates adenylyl cyclase which converts ATP to cAMP. The cAMP second messenger system is the predominant second messenger pathway stimulated by LH (Marsh, Butcher, Savard & Sutherland, 1966; Jordan *et al.*, 1978; Hoyer & Niswender, 1986), and incubation of cells with dbcAMP should mimic the steroidogenic action of LH independently of the availability of receptors.

The aim of this study was to examine basal and LH-stimulated progesterone production *in vitro* by dispersed bovine luteal cells from the 7-day old GnRH-induced CL, and to compare it to basal and LH-stimulated progesterone production by the spontaneously-formed CL of the same age. Brunswig *et al.* (1986) reported that enzymatically dispersed bovine luteal cells incubated with hCG produced six times more progesterone than that produced by unstimulated cells.

In Chapter 7, basal progesterone production by separated small and large luteal cells was low in the 24-hour period following collagenase dispersion, when compared to the study of El boudrari *et al.* (1990). It is known that the lutropin/choriogonadotrophin (LH/CG) receptor is susceptible to degradation by enzymes used in the dispersion medium (Segaloff *et al.*, 1990). Alternatively, precursors for progesterone synthesis by the luteal cells may have been limiting. Steroidogenic tissues can derive cholesterol from circulating lipoproteins *in vivo*, and this may provide the major source of substrate for steroid synthesis. Blood-borne cholesterol appears to be physiologically important for luteal function in the cow,

because elevated serum cholesterol values result in increased progesterone concentrations during the luteal phase (Talavera, Park & Williams, 1985). Circulating lipoprotein-associated cholesterol is probably the preferred substrate, as opposed to *de novo* synthesis of cholesterol in many species (see review by Gwynne & Strauss, 1982; Swann & Bruce, 1986). In bovine luteal cells cholesterol synthesized *de novo* is apparently unnecessary for progesterone synthesis when lipoproteins are present (O'Shaughnessy & Wathes, 1985b). The two major carriers of blood cholesterol in the bovine are LDL and HDL. Bovine luteal cells *in vitro* can utilize either LDL or HDL as a source of cholesterol for increased progesterone production (Pate & Condon, 1982; O'Shaughnessy & Wathes, 1985b; Pate, Nephew & Zarle, 1987; Pate & Nephew, 1988). HDL is the major lipoprotein class present in bovine serum (Jonas, 1972; Raphael, Dimick & Puppione, 1973; Chapman, 1980). The ratio of HDL to LDL varies with the lactational state of the animal and may rise to 26.7 (Raphael, Dimick & Puppione, 1973), suggesting that HDL plays an important role in providing cholesterol to the bovine CL.

O'Shaughnessy & Wathes (1985b) reported that LDL was 7 times more potent on a protein-weight basis, and 3.5 times more potent on a basis of cholesterol content, than HDL, in stimulating progesterone production. In the study of Carroll *et al.* (1992) the dose response curve showed no difference in the ability of bovine LDL *versus* HDL to stimulate progesterone production by bovine luteal cells on a cholesterol basis. However, on a protein basis LDL stimulated higher progesterone production than did HDL, because of different protein to cholesterol ratios. Comparisons of LDL and HDL on a cholesterol basis more accurately reflected the mass conversion of cholesterol to progesterone by the luteal cell (Carroll *et al.*, 1992).

An additional aim of this study was to examine whether the incorporation of HDL in the incubation medium would enhance

progesterone production by the dispersed luteal cells, and to determine the optimum dose.

8.2. Materials and Methods

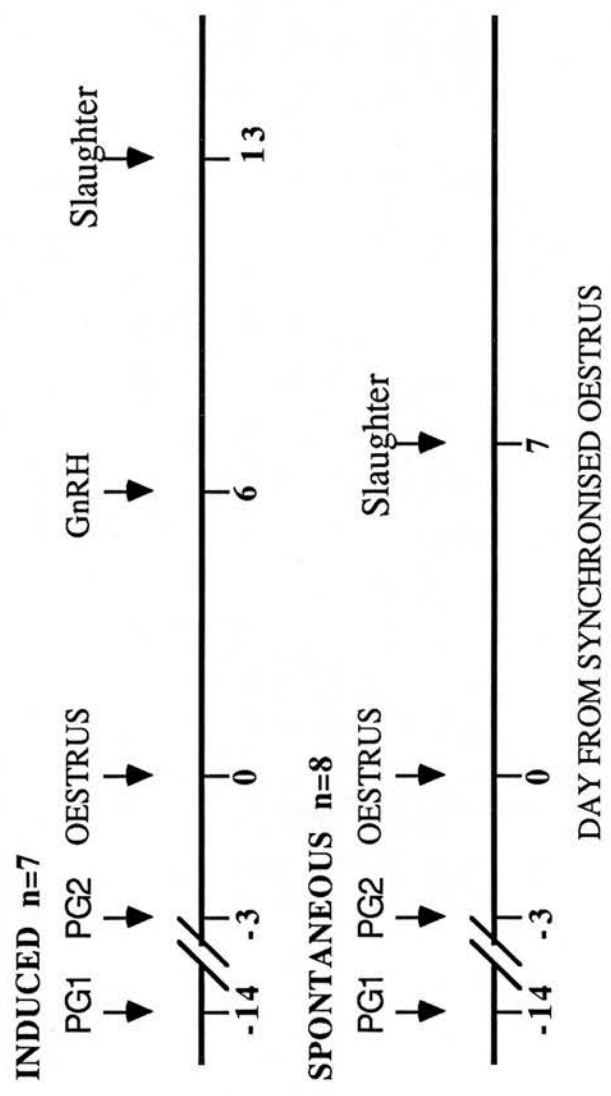
8.2.1. Animals

Mature Hereford cross Friesian heifers were housed in straw yards on the Institute Large Animal Unit and fed a maintenance ration of hay and grass nuts.

8.2.2. *In vivo* experimental procedure

The protocol for the *in vivo* experiment is shown in Figure 8.1. and is similar to Experiment 1, Chapter 5 (see section 5.2.) The oestrous cycles of 15 heifers were synchronized (oestrus = Day 0) with 2 injections of 500 ug cloprostenol (Estrumate: Coopers Animal Health, Crewe Hall, Cheshire), 11 days apart (PG1 and PG2). Oestrous behaviour following PG2 was monitored by 3 times-daily observation, together with the use of heat detection devices (Kamar Heatmount Detectors: Kamar Inc., Steamboat Springs, CO, USA). Oestrus was confirmed retrospectively by daily blood sampling for serum progesterone levels. Seven heifers received 0.5 mg GnRH (Fertagyl: Intervet Laboratories Ltd., Science Park, Milton Rd., Cambridge) by i.m. injection on Day 6 (oestrus = Day 0), and were slaughtered on Day 13. Eight untreated heifers were slaughtered on Day 7. Ovaries were collected and transferred immediately to Medium 199 containing antibiotics and sodium bicarbonate (Gibco Life Technologies Ltd., Paisley, Scotland) at 37°C for transport to the laboratory where all structures were recorded and CL dissected and weighed.

Figure 8.1.PROTOCOL FOR IN VIVO EXPERIMENT



8.2.3. Hormone radioimmunoassay

Blood samples were collected by jugular venepuncture, and serum harvested and stored at -20°C for subsequent measurement of peripheral progesterone concentrations.

8.2.3.1. Progesterone assay

Serum samples from this study were assayed for progesterone in 2 assays. The inter- and intra-assay coefficients of variation were 9.2% and 9.1% respectively.

8.2.4. *In vitro* experimental procedure

Bovine luteal cells were obtained by collagenase dispersion, as described in Chapter 2 (see section 2.8.), from spontaneously-formed and GnRH-induced CL obtained from heifers at slaughter, following a preliminary study to optimise the concentration of HDL cholesterol to be included in the incubation medium (see Figure 2.5., Chapter 2). After 30 minutes preincubation the cells were challenged with the following treatments (see section 2.11.1., Chapter 2). Each treatment was performed in triplicate. Unless otherwise stated all reagents were from Sigma Chemical Co. (Poole, Dorset, UK).

- (1) Response to LH: The doses used in this study were as follows: 0.1, 1, 10, 100 ng/ml bovine LH (USDA bLH-B-5, USDA-Reproduction Lab., Beltsville, MD 20705). Freeze-dried bovine LH was suspended in PBS to achieve the required concentrations. The dose range of LH was based on a study by Davis *et al.*, (1989) who found that

progesterone accumulation in dispersed bovine small luteal cells was maximal at 1-10 ng/ml.

- (2) Response to dbcAMP: The dose used in this study was as follows: 0.5 mg/ml dbcAMP ($N^6,2'$ -O-dibutyryladenosine-3':5'-cyclic monophosphate sodium salt) following a preliminary study to optimise the concentration of dbcAMP (see Figure 2.6., Chapter 2).

8.2.5. Progesterone assay

Media samples from this study were assayed for progesterone in 6 assays. The mean sensitivity of the assays was 10.0 pg/tube. The inter- and intra-assay coefficients of variation were 3.9% and 7.7% respectively.

8.2.6. Statistical analysis

All data are presented as means \pm sem. Baseline production of progesterone was defined as the amount of progesterone in the medium collected after the 30 minute preincubation period. Progesterone production in the 4-hour challenge period was expressed as a percentage of baseline progesterone production. Media progesterone profiles were compared using fitting effects for the different treatments, linear regression analysis on Genstat. Differences between the spontaneous and induced CL in luteal weight and baseline progesterone production were tested by Student's *t* test.

8.3.Results

8.3.1. *In vivo* observations

8.3.1.1. Peripheral progesterone concentrations

Analysis of peripheral progesterone profiles for the individual animals indicated that 1 of the 15 heifers did not undergo luteolysis in response to the double prostaglandin synchronisation regime. Concentrations of progesterone in the peripheral serum of all other animals fell below 1 ng/ml following PG2.

8.3.1.2. Response to GnRH

Five (71%) of the 7 treated heifers responded to the GnRH challenge with an additional ovulation. Mean weight of induced CL (3.8 ± 0.5 g) was significantly reduced ($P < 0.01$) when compared to mean weight of spontaneously-formed CL (6.5 ± 0.5 g).

8.3.2. *In vitro* observations

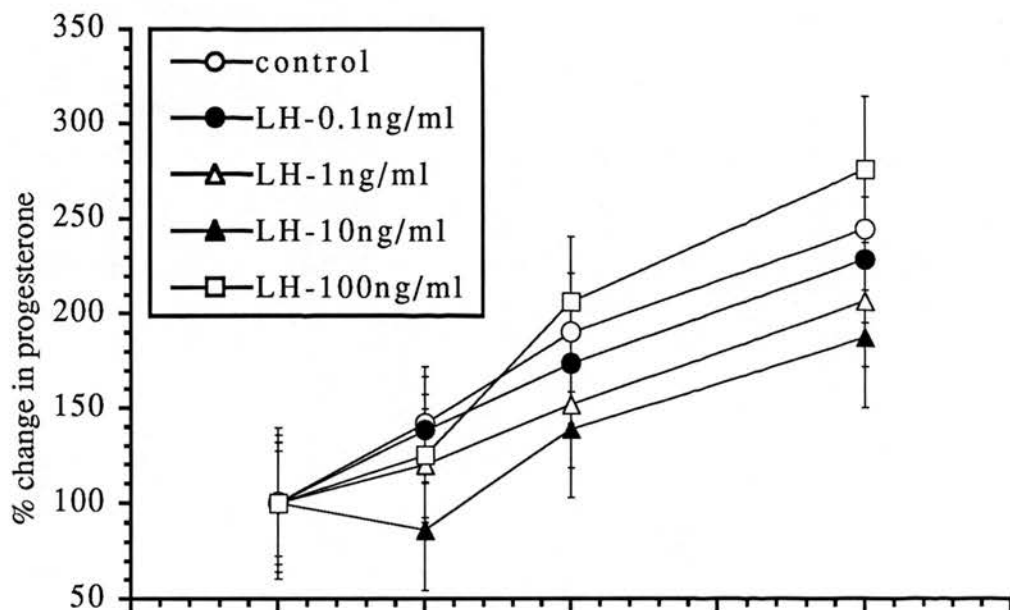
Mean baseline progesterone production for the induced CL was significantly higher ($P < 0.001$) than mean baseline progesterone production for the spontaneously-formed CL (74.5 ± 17.3 ng/ 10^6 cells and 13.4 ± 3.9 ng/ 10^6 cells respectively).

8.3.2.1. Response to LH

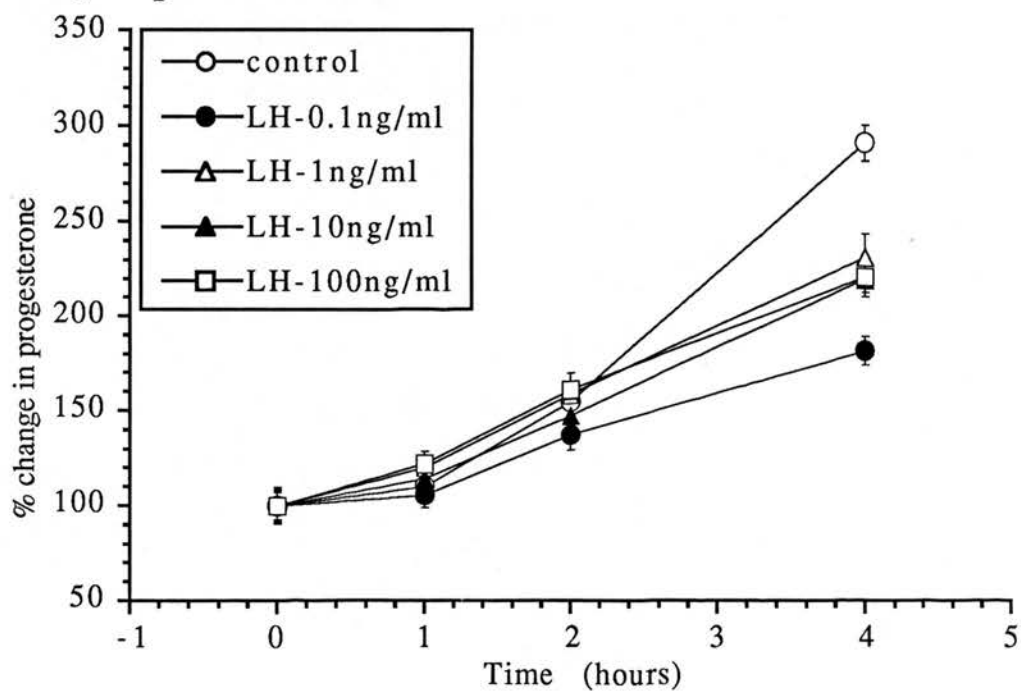
The mean (\pm sem) percentage change in media progesterone production from dispersed bovine luteal cells incubated with 0.1, 1, 10, and 100 ng/ml bovine LH for 4 hours is shown in Figure 8.2. The response of

Figure 8.2. Mean (\pm sem) progesterone production during the 4-hour incubation period by dispersed bovine luteal cells from induced CL (n=5: upper panel) and spontaneous CL (n=6: lower panel) incubated in the presence of 0.1, 1, 10, and 100 ng/ml bovine LH. Data are expressed as a % of progesterone production in the preincubation period.

a) Induced



b) Spontaneous



dispersed luteal cells from induced CL is shown in the upper panel and the response of dispersed luteal cells from spontaneous CL is shown in the lower panel. There was no significant increase in progesterone production with any of the LH treatments compared to control incubations, in either the induced CL or the spontaneous CL.

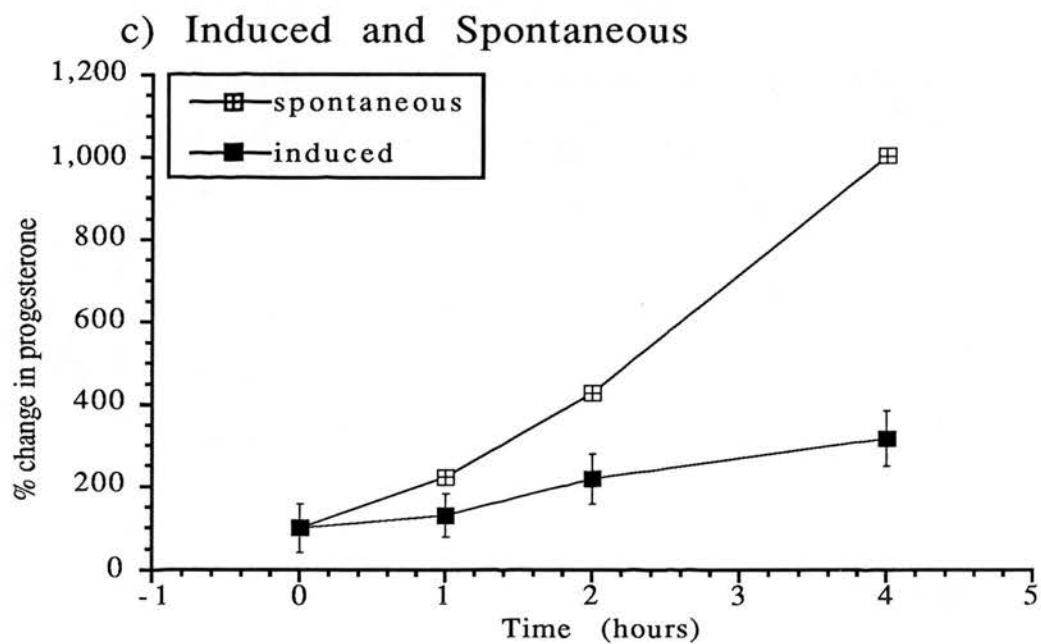
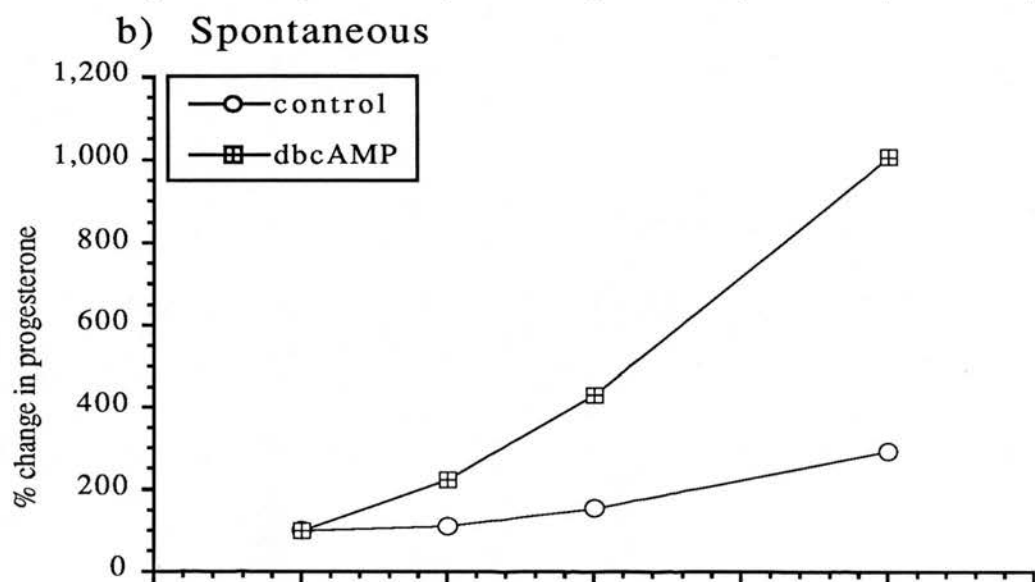
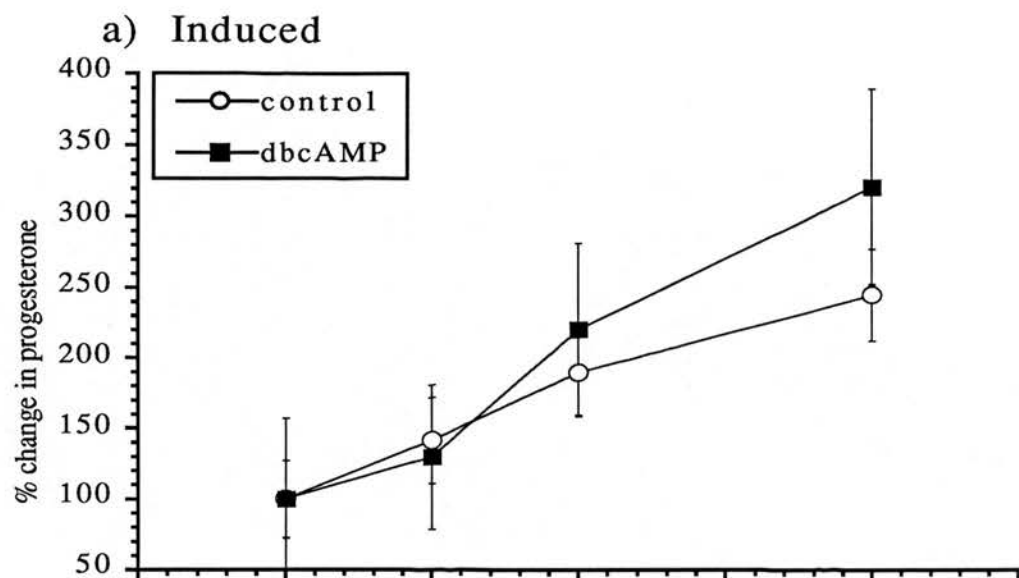
8.3.2.2. Response to dbcAMP

The mean % change (\pm sem) in media progesterone production from dispersed bovine luteal cells incubated with 0.5 ng/ml dbcAMP for 4 hours is shown in Figure 8.3. The response of dispersed luteal cells from induced CL is shown in the upper panel and the response of dispersed luteal cells from spontaneous CL is shown in the middle panel. The response of luteal cells from induced CL is compared to the response of luteal cells from spontaneous CL in the lower panel. There was no difference in progesterone production by control incubations of luteal cells from induced CL and spontaneous CL, or in progesterone production by luteal cells from induced CL in response to dbcAMP, compared to control incubations. There was a significant increase in progesterone production by the dbcAMP-treated luteal cells from spontaneous CL, when compared to the control incubations ($P < 0.05$) and to the dbcAMP-treated luteal cells from induced CL ($P < 0.001$).

8.4. Discussion

These results have shown that there is a dramatic difference in the response to dbcAMP *in vitro* by spontaneously-formed CL (4-fold increase in percentage change in progesterone production in the 4-hour period) when compared to induced CL. Both LH and dbcAMP are known to stimulate the production of progesterone by bovine luteal cells (Godkin *et al.*, 1977; Ling & Marsh, 1977; O'Shaughnessy & Wathes, 1985a). In a study by Rodgers *et al.* (1988) treatment with either LH (100 ng/ml) or dbcAMP (1 mmol/l) had no

Figure 8.3. Mean (\pm sem) progesterone production during the 4-hour incubation period by dispersed bovine luteal cells from induced CL (n=5: upper panel), spontaneous CL (n=6: middle panel) and induced CL compared to spontaneous CL (lower panel), incubated in the absence or presence of 0.5 mg/ml dbcAMP. Data are expressed as a % of progesterone production in the preincubation period. The rate of change in progesterone production was significantly ($P<0.05$) greater in the spontaneous dbcAMP group compared to the other three groups, which were not significantly different from each other. The model did not account for the majority of the variation in the data ($R^2=0.43$).



effect on progesterone secretion by early luteal phase cells but stimulated progesterone secretion 2-4 fold by cells from the later stages. The lack of response to LH by young CL was possibly due to the low numbers of LH receptors found in young CL (Rao *et al.*, 1979; Spicer *et al.*, 1981) but the lack of response to dbcAMP suggested that other components of the LH-stimulated pathway were also not fully developed. It is possible that the lack of response to dbcAMP by cells from GnRH-induced CL reflected a slower rate of maturation of the cAMP-mediated second messenger pathway. Indeed, Harrison *et al.* (1987) observed an increased sensitivity of Day 10 small ovine luteal cells to LH (compared with Day 15 of the oestrous cycle) although numbers of receptors for LH did not change between Day 10 and 15, supporting the concept that events within the cell that occur subsequent to hormone binding change during the oestrous cycle, and maybe important in modulating the magnitude of the response to LH.

In this study mean luteal weight was significantly lower in the 7-day old induced CL when compared to spontaneously-formed CL of the same age. These results are in contrast to the observations from the previous study (see Chapter 5), in which mean weights of spontaneously-formed (3.9 ± 0.8 g) and induced CL (3.5 ± 0.4 g) of the same age were not different, and similar to the mean weight of the induced CL in this study (3.4 ± 0.5 g). Hu *et al.* (1990) reported that luteal weight and *in vitro* production of progesterone did not differ between normal CL and subnormal CL (CL destined to be short-lived) 6 days after ovulation. In contrast, Kesler *et al.* (1981a) reported that bovine CL obtained 7 days after ovulation induced by GnRH were smaller and produced less progesterone *in vitro* when challenged with LH than CL removed from normally cycling cows. However, no difference in luteal weights or *in vitro* response to LH were noted when CL were collected on Day 5, suggesting that luteal development and/or maintenance beyond Day 5 was inhibited, and

that the 7-day old CL failed to respond to LH *in vitro* because luteolysis had begun. In support of these findings, Duby, Browning, Carey & Black (1985) observed that the response of luteal cells to LH stimulation appeared to be related to the histological integrity of the tissue at the time of removal. CL undergoing luteolysis (as evidenced by vacuolation of the cytoplasm, condensation of the nucleus of the large cells, pyknosis of the nucleus in the small cells and an increase in fibroblasts) did not respond to LH *in vitro*. Conversely, CL composed of large polyhedral cells with few cytoplasmic vacuoles and lightly staining nuclei were functional. In this study, although induced CL were lighter, there was no difference in *in vitro* progesterone production by control incubations of induced and spontaneously-formed luteal tissue.

Following a preliminary study using spontaneously-formed luteal tissue, it was apparent that incorporation of lipoprotein into the incubation medium (200 µg HDL/ml) dramatically increased progesterone production by dispersed luteal cells (5.5-fold increase in progesterone production over controls in the 4-hour incubation period). Hunter *et al.* (1988) observed that luteal tissue from short-lived CL responded to the supplementary supply of precursor (pregnenolone) *in vitro* with increased progesterone production, suggesting that subnormal progesterone production may be caused by an inadequate supply of substrate.

Dibutyryl cAMP may act to maintain cholesterol side-chain cleavage activity since cultured cells maintained under basal conditions were unable to respond to dbcAMP to the same extent as cells maintained in dbcAMP throughout culture (O'Shaughnessy & Wathes, 1985a). In addition, these workers observed a small stimulation of progesterone production by LH although the degree of stimulation was less than that observed in response to dbcAMP. In the absence of trophic stimulation, progesterone secretion

declined rapidly during culture while addition of dbcAMP greatly diminished the rate of decline. Furthermore, it has been shown that dbcAMP increases cholesterol side-chain cleavage activity in cultured bovine granulosa cells (Funkenstein, Waterman, Masters & Simpson, 1983).

Conversion of cholesterol to pregnenolone is the first and rate-limiting step of steroidogenesis. The reaction is catalysed by the mitochondrial cholesterol side-chain cleavage (scc) enzymatic system, composed of the cytochrome P450_{scc} and the electron donor proteins adrenodoxin (ADX) and ADX reductase (Miller, 1988; Simpson & Waterman, 1988). In the bovine CL, P450_{scc} and ADX are induced *in vivo* by the LH surge and remain high during the luteal phase (Rodgers, Waterman & Simpson, 1986b, 1987). The reduced magnitude of the exogenous GnRH-induced LH surge (50-60% of the spontaneous surge: Webb, 1977; Zolman *et al.*, 1973) may have limited the induction of the components of the mitochondrial cholesterol scc enzymatic system in the GnRH-induced CL, resulting in the observed failure of dbcAMP to increase progesterone production in the cells from induced CL.

Mean baseline progesterone concentration was markedly (5.5-fold) increased in incubations of cells from induced CL when compared to cells from spontaneously-formed CL. Mee *et al.* (1993) reported that administration of GnRH 12 hours after first detected oestrus increased the proportion of large, and decreased the proportion of small luteal cells in the CL by day 10 after treatment. Luteal tissue collected from cows without previous exposure to GnRH had a greater response to LH in the *in vitro* incubation system than luteal tissue previously exposed to GnRH. These workers suggested that GnRH may have acted on the developing CL to promote the conversion of small luteal cells to large luteal cells, thereby decreasing the number of LH-responsive small luteal cells in the CL, and

reducing progesterone production in response to LH *in vitro*. However, although approximately 85% of basal progesterone secretion is attributed to the large luteal cells (Niswender *et al.*, 1985) the GnRH-induced increase in large luteal cell number was not reflected by a corresponding increase in baseline progesterone concentration (Mee *et al.*, 1993). In contrast, in this study baseline progesterone production in the GnRH-induced CL was increased in the preincubation period, when compared to the spontaneous CL, although there was no difference in the percentage change in progesterone production by control incubations from induced and spontaneously-formed CL in the 4-hour incubation period. Grazul, Kirsch, Slinger, Marchello & Redmer (1989) reported that basal secretion of progesterone by bovine luteal cells *in vitro* was greater on Day 4 than on Days 8, 14 and 18 of the oestrous cycle, in agreement with other studies (Milvae & Hansel, 1983; Harrison *et al.*, 1987; Rodgers *et al.*, 1988). Thus hormone production by luteal cells *in vitro* differs significantly with stage of luteal development.

As a consequence of the difference in mean baseline progesterone production, the actual amount of progesterone released was greater in control incubations of induced luteal cells than in control incubations of spontaneously-formed luteal cells. Furthermore, the actual amount of progesterone released by induced and spontaneously-formed luteal cells in response to dbcAMP was similar. It is possible that there could be differences in storage and/or release of progesterone by induced and spontaneously-formed CL.

In this study bovine LH, at concentrations ranging from 0.1 to 100 ng/ml, had no effect on progesterone production by luteal cells from spontaneously-formed or induced CL *in vitro*. We cannot exclude the possibility that the bovine LH had become denatured in preparation. This is

unlikely because the same preparation (USDA bLH-B-5) was used to examine the response of cultured bovine luteal cells to LH, and gave marked increases in progesterone production both in the presence and absence of bovine follicular fluid fractions (see Chapter 7). However the maximum dose used in this study (100 ng/ml) was less than the dose used in the study in Chapter 7 (1 μ g/ml). It is unlikely that the lack of response to LH reflects a reduction in cell viability post-dispersion, because the dispersed luteal cells from spontaneously-formed CL showed a dramatic increase in progesterone production in response to dbcAMP, and cell viability, as assessed by trypan blue exclusion, was >95%. The relative insensitivity of luteal cells to LH compared to dbcAMP may be due to a loss of LH receptors, a loss of adenylate cyclase activity or possibly an increase in phosphodiesterase activity.

The LH/CG receptor is readily susceptible to proteolysis by enzyme(s) present in collagenase preparations typically used to disperse gonadal tissues (Segaloff *et al.*, 1990). This degradation is dependent on the concentration of collagenase used and the time of exposure to collagenase (Ascoli & Segaloff, 1986). Collagenase concentration (2000 U/g luteal tissue) and time of exposure (2.5 hours) were based on previous *in vitro* studies utilizing dispersed luteal cells (Simmons *et al.*, 1976; Harrison *et al.*, 1987). Ascoli & Segaloff (1986) suggest that collagenase (specifically enzymes that contaminate collagenase preparations) "nicks" the LH/CG receptor and that, normally, the nicked receptor is held together by disulphide bonds. However, in contrast to this study in which LH had no effect on progesterone production, collagenase-treated cells bound hCG with normal affinity and stimulated steroid production with normal efficacy (Ascoli & Segaloff, 1986). Furthermore, endogenous proteases also degrade or nick the LH/CG receptor (Kellokumpu & Rajaniemi, 1985), but as with the collagenase-treated receptor, LH/CG receptor exposed to these proteases also

bound hCG. The proteolytic effects of collagenase could be inhibited by the metal chelating agent, EDTA (Ascoli & Segaloff, 1986). Inhibition of the proteolysis of the LH/CG receptor by endogenous proteases required EDTA and also N-ethylmaleimide and phenylmethanesulfonyl fluoride (Kellokumpu & Rajaniemi, 1985). EDTA was not included in the enzyme solution (see section 2.8., Chapter 2) as it may affect cell viability and inhibits collagenase activity.

Interestingly, for the *in vitro* studies in Chapter 7, the 4-hour challenge period which followed a 24-hour plating-down period was preceded by collagenase dispersion of the luteal cells (2000 U/g luteal tissue for 2.5 hours). These observations suggest that responsiveness to LH was reacquired in the 24-hour plating-down period. Responsiveness of a target cell to LH can be modulated by one or both of two general kinds of mechanisms. LH receptors can be down-regulated which results from an increase in the internalization and degradation of the hormone-receptor complex (Lloyd & Ascoli, 1983; Ascoli, 1984; Ascoli & Segaloff, 1987), or there may be alterations in the 'functional' activity of the receptor which can occur independently of (or in addition to) changes in receptor numbers. These mechanisms are reversible.

In conclusion, this study has demonstrated that the steroidogenic response to dbcAMP by luteal tissue from 7-day old GnRH-induced CL is markedly reduced when compared to the response by luteal tissue from spontaneously-formed CL of the same age, by an as yet unknown mechanism. Further studies are required to refine the cell dispersion technique and enable comparison of the hormone-dependent response of spontaneously-formed and induced luteal tissue, and/or locate the point(s) in the second messenger pathway responsible for the failure of the steroidogenic response in the induced CL.

CHAPTER 9

GENERAL DISCUSSION

In summary, this series of studies has examined the formation, function and regression of the induced CL. We have shown for the first time that administration of GnRH to heifers in the early luteal phase of the oestrous cycle results in ovulation and the formation of an additional CL in >70% of animals (Chapters 3 and 4). This demonstrates that the dominant follicle of the first wave of follicular growth is competent to ovulate and form a functional CL in response to a surge release of LH. Formation of the GnRH-induced CL occurs against a background of luteal phase concentrations of progesterone when the frequency of pulsatile LH secretion is low, in contrast to the CL of the spontaneous cycle which is formed at the end of the follicular phase, characterized by a high frequency, low amplitude pattern of LH secretion. In contrast, both the CL formed following regression of the endogenous CL and ovulation of the first dominant follicle as a result of administration of exogenous $\text{PGF}_{2\alpha}$ in the $\text{PGF}_{2\alpha}$ -responsive phase of the early luteal phase (Parfet, Smith, Cook, Skyer, Youngquist & Garverick, 1989), and the CL formed following administration of GnRH during seasonal anoestrus in sheep or the early post-partum period in cattle, develop in an endocrine environment characterized by low (<1 ng/ml) follicular phase concentrations of progesterone. All cattle in these studies were sexually mature, cycling heifers; thus, the GnRH treatment cycle was preceded by an oestrous cycle of normal duration and a luteal phase profile of progesterone secretion. In contrast, progesterone

concentrations preceding the formation of the first, short-lived post-partum CL in cattle, are low. However, pretreatment with progesterone increased the proportion of GnRH-induced CL with a normal lifespan (post-partum cattle: Rutter *et al.*, 1985; anoestrous sheep: Hunter *et al.*, 1988). This suggests that the mechanism(s) limiting the lifespan of GnRH-induced CL in post-partum cattle and anoestrous sheep is different to that in the GnRH-induced CL in these studies.

No differences in characteristics of the dominant follicle, monitored by ultrasound, were detected in the interval between spontaneous ovulation and GnRH administration for GnRH responders and non-responders, which could account for the non-response rate (<30%). Furthermore, the growth rates and maximum sizes of the dominant follicle of the first (anovulatory) and third (ovulatory) waves of follicular growth in the natural bovine oestrous cycle were similar (Sirois & Fortune, 1988). However, some alterations in development were detected in follicles destined to form abnormal CL, compared to those destined to form normal CL in cattle (reduced oestradiol in follicular fluid; reduced LH and FSH receptors; and an increased granulosa cell numbers; Braden, King, Odde & Niswender, 1989b).

The functional capacity of the induced CL was assessed both *in vivo* and *in vitro*. The progesterone profiles of GnRH responders indicated that the induced CL was indeed functional, although progesterone production by the induced CL was relatively low compared with that of the spontaneously-formed CL (Chapter 3). However, in a subsequent study (Chapter 5), in which luteal tissue was collected at slaughter on Day 7 after the GnRH-induced ovulation, progesterone profiles of animals with induced or spontaneous CL were not different in the interval between luteolysis of the endogenous CL in the GnRH-treated group and slaughter. By classifying induced CL as functional if mean serum progesterone concentrations exceeded 1 ng/ml

before the demise of the induced CL (Chapter 4), it was apparent that the proportion of GnRH responders with a functional induced CL was very variable (approximately 65%), whereas Southee, Hunter & Haresign (1988) reported that approximately 70% of GnRH-induced CL in anoestrous ewes were abnormal, with a transient rise in plasma progesterone to >0.5 ng/ml followed by a rapid decline. Also Walters, Short, Convey, Staigmiller, Dunn & Kaltenbach (1982) reported a 55% incidence of short cycles after pulsatile GnRH injection and a 64% incidence after weaning in post-partum beef cows.

By manipulating the endocrine environment (specifically by reducing serum oestradiol concentrations with the administration of bFF), it was possible to increase progesterone production by the induced CL (Chapter 4). However progesterone production was still limited when compared to the spontaneously-formed CL, and the results of a subsequent study (Chapter 7) demonstrated that bFF contained a factor(s) that inhibited progesterone production by mixed populations of cultured luteal cells *in vitro*. The inhibitory effect was seen with both a steroid-stripped, inhibin-enriched fraction and steroid-stripped, inhibin-depleted fractions of bFF. We have suggested that the administered bFF has a direct inhibitory effect on progesterone production by the induced CL, which is apparent in the first 4 days of administration, when serum oestradiol concentrations are similar between groups, but serum progesterone concentrations are reduced in the bFF-treated group (Chapter 4). As referred to below, there is circumstantial evidence that oestradiol may be responsible for the regression of the induced CL. However, there was no difference in the total oestradiol concentrations to which functional and non-functional induced CL were exposed, prior to the start of the treatment period (Chapter 4). We have suggested that differences in hypothalamo-pituitary sensitivity to the

feedback effects of oestradiol secreted by the dominant follicle of the second follicular wave in the endogenous cycle, or differences in luteal and/or endometrial sensitivity to the luteolytic effect of oestradiol, between animals, determined whether the induced CL was functional.

Finally, in Chapter 6, we demonstrated that progesterone production by the induced CL was adequate to prime the uterus to respond to an exogenous oxytocin challenge with a release of PGF₂ α of similar magnitude to that observed in control animals with a spontaneously-formed CL of the same age.

Functional capacity of the induced CL was assessed *in vitro*. On Day 7 after ovulation mean CL weights and tissue oxytocin and progesterone concentrations of induced and spontaneous CL were similar. These observations are in contrast to studies with the GnRH-induced CL in postpartum cattle and seasonally anoestrous sheep, in which there is evidence for impaired luteal function by Day 7 after ovulation (cattle: Kesler *et al.*, 1981a; Rutter *et al.*, 1985; sheep: Hunter *et al.*, 1988). There was no evidence of oestradiol production by the induced CL, in agreement with previous studies on the bovine CL of the natural cycle (Henderson & Moon, 1979). However, the concentration of LH receptors was increased in induced CL. The significance of this increase *in vivo* in relation to luteal progesterone secretion is difficult to interpret, as peripheral progesterone concentrations in the GnRH responders on the day of tissue collection were supplemented by progesterone from the endogenous CL. Furthermore, Dickman *et al.* (1978a) reported that <1% of LH receptors are occupied under conditions of maximal steroid secretion. However, following PG3 to remove the endogenous CL, concentrations of LH receptors in induced and spontaneous CL were similar, and peripheral progesterone concentrations were not significantly different (Chapter 5).

Finally, in Chapter 8, it has been demonstrated that the steroidogenic response *in vitro* of luteal tissue from 7-day old GnRH-induced CL to dbcAMP is markedly reduced when compared to the response by luteal tissue from spontaneously-formed CL of the same age, although total progesterone production was similar. We have suggested that the reduced magnitude of the exogenous GnRH-induced LH surge (50-60% of the spontaneous surge: Webb, 1977; Zolman *et al.*, 1973) may have limited the induction of the components of the mitochondrial cholesterol side-chain cleavage enzymatic system in the GnRH-induced CL, resulting in the observed failure of dbcAMP to increase progesterone production in the cells from induced CL.

Regression of the GnRH-induced CL occurs prematurely when compared to the spontaneously-formed CL (Chapter 3). Although luteal lifespan was extended by the strategic use of bFF to suppress the growth of the dominant follicle during the development of the induced CL, the function of the induced CL was limited when compared to the spontaneous CL (Chapter 4). These results support the hypothesis that the CL induced by the administration of GnRH in the early luteal phase does not control its own lifespan. Rather, its demise appears to be associated with an endogenous signal from the dominant follicle. The bFF-induced alteration in lifespan of the induced CL is independent of changes in mean serum gonadotrophin concentrations. However, the bFF-induced reduction in mean serum oestradiol concentrations and the subsequent increased secretion of oestradiol coincident with the premature demise of the induced CL and the decline in peripheral progesterone concentrations observed in Experiment 3 (Chapter 4) provide circumstantial evidence that oestradiol is the endogenous agent responsible for regression of the induced CL.

The absence of a decrease in LH binding indicates that the short lifespan of the induced CL is not a consequence of the lack of the ability to respond to luteotrophic support. Furthermore, a study comparing the oxytocin-induced PGFM response in animals with induced and spontaneous CL of the same age (Chapter 6), has indicated that the premature demise of the induced CL does not appear to be the result of oxytocin-induced reduction in luteotrophic support, or of advanced release of $\text{PGF}_{2\alpha}$. This is in contrast to the short-lived CL, in which luteolysis has been shown to be the result of the early release of a luteolytic pattern of $\text{PGF}_{2\alpha}$ secretion from the uterine endometrium (Zollers *et al.*, 1989, 1991). Thus, in this series of studies, the luteolytic effect of oestradiol appears to be independent of the induction of endometrial oxytocin receptors and oxytocin-induced release of $\text{PGF}_{2\alpha}$. It is possible that oestradiol is having a direct effect on the induced CL. Oestrogen receptors are present in the sheep CL (Glass *et al.*, 1984), and a specific oestradiol-17 β binding protein has been identified in the cytosol fraction of the bovine CL (Kimball & Hansel, 1974).

In conclusion, these studies have provided an insight into the factors which determine the formation, function and regression of the GnRH-induced CL. This induced CL is unique. It is formed following ovulation of the first dominant follicle in a progesterone-dominated environment, and is exposed to a potentially luteolytic dose of $\text{PGF}_{2\alpha}$ soon after formation, followed by a dramatic change in the endocrine environment with the rapid decline in peripheral progesterone concentrations as a result of the demise of the endogenous CL, and the emergence of the (oestrogenic) dominant follicle of the second follicular wave of the endogenous cycle. Progesterone production is limited, both in the absence and presence of bFF to reduce peripheral oestradiol concentrations, and luteal cell responses to dbcAMP *in vitro* indicate a failure of the steroidogenic response at a point(s) distal to

second messenger formation. Regression of the induced CL is premature and these studies suggest that the induced CL does not control its own lifespan, but that its demise appears to be associated with an endogenous signal from the dominant follicle. The clinical significance of a better understanding of the relationship between the CL and the dominant follicle in future oestrous synchronization programmes justifies further investigation into the factor(s) which control the ephemerality of the CL.

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